THE UNIVERSITY *of* EDINBURGH

Application of novel methods using synthetic biology tools to investigate solvent toxicity in bacteria

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2014

Doctor of Philosophy (PhD) – The University of Edinburgh – 2014

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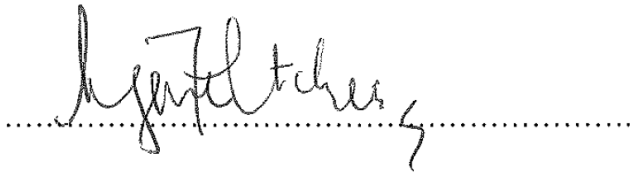
Abbreviations

Abbreviation	Meaning
%	Percent
^{o}C	Degree Celsius
µg	microgram
µl	microliter
CML	Chloramphenicol
DNA	Deoxyribonucleic Acid
et al.	et allii (and others)
g	gram
EGFP	Enhanced Green Fluorescent Protein
hr	hour
IPTG	Isopropyl thiogalactopyranoside
LB	Lysogeny Broth
M	Molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
PCR	Polymerase Chain Reaction
RBS	Ribosome Binding Site
RFP	Red Fluorescent Protein
RNA	Ribonucleic acid
sec	seconds

Declaration

This thesis sums up research done by me and unless otherwise stated, all the results present herein are my own work. This work has not been submitted for any other degree or personal qualification.



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2014

Acknowledgements

I would like express my sincerest gratitude to Dr Chris French, my PhD supervisor, for the guidance, direction and mentorship I received from him throughout the course of my PhD. I also owe a debt of gratitude to Drs Garry Blakely, Maurice Gallagher and Andrew Free for keeping me on track and helping my project to take shape. I always learnt a lot from them during those thesis committee meetings we had. I would also want to specially thank the Darwin Trust of Edinburgh for the financial support I got through the benevolence of Professors Kenneth and Noreen Murray. May their souls rest in perfect peace! Had it not been for the Trust, I will never have been able to pay for the education I received here in Edinburgh.

Many thanks to Dr Teuta Pilizota for all the help with the fluorescence microscopy and for offering the EGFP construct I used for my work. To Dr Nimisha Joshi, I say 'thank you' for allowing me to use the oxidative stress response genes you made, for my work. My appreciation goes to the 2010 TU-Delft iGEM team for generously sending me the Prefoldin construct. I deeply appreciate the contribution of all these people to my work.

Special thanks go to my parents, Isaac and Rita, and the rest of my family for their support and encouragement. My final word of gratitude goes to all the friends I made here and to all the lovely Scots I met, for making Scotland a second home!

Nyame ne hene!

Abstract

Toxicity of organic solvents to microbial hosts is a major consideration in the economical production of biofuels such as ethanol and especially butanol, with low product concentrations leading to high recovery costs. The key to rational engineering of solvent tolerant microorganisms for such processes lies in obtaining appropriate tolerance genes (modules) suited for different compounds. In this project, a synthetic biology approach was adopted to generate a library of standardised BioBrick parts involved in different stress responses. Using a multiple-assay approach, including a bioluminescence assay, these stress response genes were tested individually and in combination to determine their effects on survival in ethanol, *n*-butanol, acetone and fermentation inhibitors produced by biomass pre-treatment. A set of tolerance modules was obtained for ethanol and *n*-butanol. Proof-of-concept tests suggested that ethanol and *n*-butanol toxicity was mainly due to damage to membrane, cellular proteins and DNA possibly by oxidative stress. No synergistic interactions were observed from a combination of different tolerance genes. Further tests carried out using enzyme and fluorescence-based assays to elucidate the effect of *n*-butanol on the cell envelope showed that the solvent released lipopolysaccharides from the outer membrane of *E. coli* and also caused both outer and inner membranes to be leaky. Very high *n*-butanol concentrations resulted in an altered cell shape and bleb formation suggesting an impairment in cell division and peptidoglycan biosynthesis. The cell membrane was modified by *cis-trans* isomerisation of unsaturated fatty acids in the phospholipids resulting in a reduction of membrane leakage which in effect, increased *n*-butanol tolerance in *E. coli*. In conclusion, results from this research suggest that strategies to protect the membrane and cellular proteins should be included in rational engineering of *n*-butanol tolerant bacteria.

Chapter 1: Introduction and Literature Review

1.1 Introduction

The conversion of lignocellulosic biomass into bioethanol, butanol and other useful liquid fuels has become an urgent global priority not only because of the abundance of cellulosic biomass, but also because such fuels are environmentally friendly. The low conversion rate and the inability of the biocatalysts to tolerate high concentrations of the organic product make this process unprofitable. In order to produce high concentrations of the product it is important that the growth of the micro-organisms involved in the bioconversion is not inhibited by the toxicity of the product.

Some micro-organisms possess natural mechanisms which protect them from the toxicity of organic solvents. These include efflux pumps which extrude these solvents out of the cell, cell membrane modification, the production of protein chaperones which protect other cellular proteins, among others. A few of these mechanisms have been well-studied (Ramos *et al*. 2002) providing important cues that can be picked from these natural mechanisms to effectively engineer micro-organisms to survive in high concentrations of organic solvents during production.

Synthetic biology provides a feasible approach to engineering solvent tolerance phenotypes in micro-organisms by using tools such as BioBricks (French, 2009). Some genes implicated in organic solvent tolerance, for example *glpC* and *robA*, have been discovered (Shimizu *et al*. 2005b) even though the exact mechanism by which they act is not fully understood. BioBrick parts can be made from genes responsible for the various mechanisms of solvent tolerance, such as efflux pumps,

cell-membrane modification factors and protein chaperones which protect cellular proteins from damage, together with their transcriptional activators and then assembled into a genetic circuit to provide some tolerance to organic solvents. Testing the individual effects of these parts and a combination of parts from different protective mechanisms might prove essential in developing solvent tolerant microorganisms. The different mechanisms might interact in synergy bringing about a resultant increase in solvent tolerance. The modular and interchangeable nature of BioBricks provides an ideal platform for testing different combinations of genes. Furthermore, it will be interesting to employ organic solvent tolerant bacteria as microbial factories to produce next generation fuels and high value compounds since the biosynthetic pathway of these chemicals can also be designed and assembled using different BioBrick parts in these micro-organisms.

In this first chapter, literature elaborating the process of converting biomass into liquid fuels, reasons why organic solvents including liquid fuels are toxic and the stress responses whenbacterial cells are exposed to these fuels will be discussed. Recent attempts that have been made using novel genetic approaches to unravel new genes and mechanisms of solvent toxicity will also be reviewed. Even though so much is currently known about the genes that respond to solvent-induced stresses, the outcome of increasing solvent tolerance in model organisms is still appalling primarily because the contribution of individual stress response genes to tolerance is still not well-understood. With this in mind, I have suggested a proposition of using a synthetic biology approach and novel methods to screen stress response genes against different organic solvents with the intention of further understanding the effect of the solvents on microbial hosts which is summed up in the aims of this PhD research. In the subsequent chapters, the methods used to answer the questions raised

in this research will be shown, followed by results obtained from screening a library of stress response genes. The outcome of an investigation of the effect of *n*-butanol on the cell envelope of *Escherichia coli* (*E. coli)* and the effect of membrane modification by fatty acid *cis-trans* isomerisation on *n*-butanol tolerance will also be presented. This thesis will end with a final chapter to highlight key results and future work that can be done to extend this piece of research.

1.2 Biomass conversion into biofuels

Next generation biofuels, mainly biobutanol, isopentenol, pinene, among others, have received a lot of attention in recent times as a suitable alternative to fossil fuels which are already fast depleting. There is also no doubt that societies will gradually be shifting away from the use of fossil fuels not only because of increasing oil prices but also because of the harm they cause to the environment. Biofuels are obtained from biomass in either chemical and/or biological processes. Unlike the chemical process, biological conversion of lignocellulosic biomass to different organic solvents does not require a lot of energy and the process involves the fermentation of glucose units obtained from the hydrolysis of the biomass into different organic solvents. Ethanol happens to be the major component of the biofuels used by the USA and Brazil. As such, biofuel production has mainly focussed on ethanolic fermentation. Moreover, ethanol can be blended with gasoline and used to power engines of cars and other equipment (Lynd *et al*. 1991). Butanol, a four-carbon alcohol, is superior to ethanol as a biofuel in many ways – it has a higher energy content, lower volatility, is less hygroscopic and less corrosive (Durre, 2007). Plus, butanol has similar characteristics to gasoline and can be used in a gasoline engine without the need for modification (Savage, 2011). Despite these advantages, biobutanol production has not received as much attention as ethanol production has.

Biological conversion of lignocellulosic biomass involves four main steps. First among these is the production of cellulases and hemicellulases. These are required in the next step to hydrolyse the cellulose fibres into a mixture of sugars. The third and fourth steps involve the fermentation of the hexoses and pentoses respectively (Lynd *et al*. 2005). Currently, the production of biofuels from feedstock has not been lucrative due to low yield thus making this process economically unfavourable. A meagre 50 gallons of ethanol is produced per dry tonne of biomass (Greer, 2005) necessitating the attempts being made to increase the yield from bioprocessing. According to Lynd *et al*. (2005), in order to reduce operational costs and improve yield at the same time, it is necessary to string the individual processes of bioconversion into a single chain in a suitable organism in what is called Consolidated Bioprocessing (CBP). A separate review by French (2009) highlights the importance of creating an Ideal Biofuel Producing Microorganism (IBPM) which can convert cellulose into simple sugars, ferment these into liquid fuels in high concentrations and finally be able to rapidly grow in a bioreactor might be the way forward to efficiently converting biomass into liquid fuels. In either case (CBP or IBPM), emphasis is placed on higher efficiency and reducing costs involved in bioprocessing.

1.3 Biofuel production in *E. coli*

Naturally, certain species of *Clostridium*, *C. acetobutylicum* in particular, are able to produce butanol and are actually used industrially for commercial production of this alcohol (Lin and Blaschek, 1983). However, *C. acetobutylicum* is a gram positive, anaerobic spore forming bacterium which is fastidious and has peculiar nutritional needs (Louis *et al*., 2004) making it challenging to use this organism in biobutanol production on a large scale. It also produces ethanol and acetone as by-products of

the butanol biosynthesis pathway. Again, the characteristic slow growth and lack of efficient molecular tools to engineer *C. acetobutylicum* poses further problems with using this organism as a preferred biofuel host (Atsumi *et al*., 2008)

There has therefore been the need to identify and engineer other microbial hosts for the production of biofuels. Recently, *E. coli*, a gram negative, rod shaped facultative anaerobic bacterium found in the gut of warm-blooded animals has been considered as a host (chassis) of choice to produce biofuels following whole genome sequencing of the LCT-EC106 strain (Li *et al*., 2012). *E. coli* is probably the most studied prokaryote and it application in biotechnology as a model organism dates back to the early 1970s where Cohen *et al*. (1973) successfully transferred a functional plasmid DNA into the cell. The *E. coli* genome was one of the earliest to be sequenced revealing the presence of 4288 protein-coding genes (Blattner *et al*., 1997). Since then, its genome and metabolism has been studied in detail. This bacterium is easily manipulated for the purposes of genetic engineering and molecular tools for its manipulation abound. Again, its high growth rate with no strict nutritional requirements makes it suitable for biotechnology applications. Furthermore, since it possesses numerous metabolic pathways, these pathways can be tweaked for different applications. The availability of molecular tools has made it possible to engineer different biofuel biosynthesis pathways in *E. coli* even though it does not produce biofuels naturally. Atsumi *et al*., (2008) successfully transplanted the 1-butanol biosynthesis pathway from *C. acetobutylicum* to *E. coli* demonstrating the possibility of producing biofuels in non-native hosts such as *E. coli*. Table 1.1 shows other biofuels that have been produced in engineered *E. coli* and the maximum titres obtained.

Table 1.1 Production of biofuels in engineered strains of *E. coli*

Biofuel	Maximum titre	Reference
Ethanol	45 g/l	Jarboe *et al*., 2007
1-butanol	30 g/l	Shen *et al*., 2011
Isopropanol	143 g/l	Inokuma *et al*., 2010
Farnesene	380 mg/l	Wang *et al*., 2011
Isobutanol	50 g/l	Baez *et al*., 2011
Medium chain fatty alcohol	0.1 g/l	Steen *et al*., 2010
Biodiesel	1.28 g/l	Kalscheuer *et al*., 2006

It must be pointed here that one major hurdle involved in producing biofuels in microorganisms is toxicity of the final product. Although *E. coli* was successfully engineered to produce biobutanol, its growth was severely impaired by 7.5 g/l of the final product (Atsumi *et al*., 2008). *C. acetobutylicum* naturally produces butanol but cannot survive beyond an inhibitory concentration of 13 g/l of butanol (Jones and Woods, 1986). There have been suggestions to further increase biofuel production in *E. coli* and other organisms by enhancing metabolic pathways. Even though this comes across as a plausible idea, increasing yield and titre clearly goes beyond fine-tuning metabolic pathways. It is thus essential to employ a robust microorganism that can survive the toxicity of these products of biomass conversion in bioprocessing.

Using *E. coli* as a model organism to understand the mechanisms of toxicity of organic solvents to bacteria will be beneficial in identifying new ways to increase tolerance and this can in the long term, be applied to other related organisms.

1.4 The *E. coli* cell envelope

The first point of contact of bacterial cells such as *E. coli* to the toxic compounds in its environment is the cell membrane. The cell envelope is probably the first line of defence against toxic compounds and is a crucial compartment for the cell's survival. Therefore understanding the structure and functions of the components of the cell envelope will be important in devising appropriate strategies to protect the cell from a harsh environment.

The cell envelope of gram negative bacteria such as *E. coli* is made up two membranes (an outer and inner) and a periplasmic space between these two membranes. Among other things present in the periplasm is a thin peptidoglycan layer (Figure 1.1). Both outer and inner membranes are made up of lipid bilayers and several proteins but differ a lot in their structure and composition.

The inner membrane is in direct contact with the cytoplasm and periplasm. It forms a highly selective barrier preventing external molecules from entering the cytoplasm apart from those molecules which have specific transporters embedded in the membrane. It is made up of a bilayer composed of phospholipids and proteins (Ruiz *et al.*, 2006). The phospholipids are made up of a polar, hydrophilic head and hydrophobic tail. The polar head of the bilayer faces outside and interacts with the aqueous environment exposed to that side of the membrane while the non-polar lipid

tail in each layer faces the core of each layer. The composition of phospholipids varies from organism to organism. The most abundant phospholipid in the membranes of *E. coli* cells in the exponential phase of growth is phosphatidylethanolamine (PE) making up 75% of all phospholipids in the membrane. Phosphatidylglycerol (PG) makes up 20% and the remaining 5% is cardiolipin (Dowhan, 1997). Apart from forming a formidable barrier that prevents metabolites with the cell from leaking out, phospholipids such as PE have been described as a non-protein molecular chaperone (Bogdanov and Dowhan, 1998). Phosphatidylethanolamine has been shown to assist in correct folding of an *E. coli* lactose permease (LacY) in the inner membrane of the cell. This work supports a growing body of evidence that proper folding and maturation of some membrane-associated proteins are facilitated by specific lipids. Altering the levels of PE and cardiolipin in the membrane has been used by some organisms as an adaptation to organic solvent stress (Isken and de Bont, 1998). Also, when cells are exposed to toxic compounds, depending on the chemical nature of the compound, they display an adaptive mechanism by altering the composition of the phospholipid head group by altering the relative amount of PG to PE. These changes decrease the permeability of the membrane and limit the amount of the solvent entering the cell (Isken and de Bont, 1998; Weber and de Bont, 1996)

There is an array of proteins associated with these lipids in the inner membrane. Many of them are integral and embedded within the membrane structure. These proteins include transporters and enzymes that play roles in energy metabolism, protein synthesis, lipid synthesis and cell division. Another type of protein, lipoprotein, is only attached to one of the inner membrane leaflets for example, GlpC

which anchors the *sn*-glycerol-3-phosphate dehydrogenase enzyme to the membrane (Cole *et al*., 1988). GlpC is a cysteine-rich 44 kDa protein (Cole *et al*., 1988) and has been shown to increase tolerance to hexane and cyclohexane probably by modifying the surface properties of the cell (Shimuzu *et al*., 2005). Its involvement in butanol tolerance is yet to be investigated.

Gram negative bacteria have an extra membrane on the outermost part of the cell envelope which is in direct contact with the cell's environment. This outer membrane bilayer is structurally different from the inner membrane. The first striking feature is that it is asymmetric with phospholipids on the inner leaflet, whereas the outer leaflet which is in contact with the environment is entirely free of phospholipids and is mainly made up of lipopolysaccharide (LPS) (Kamio and Nikaido, 1976). The lipopolysaccharides have negatively charged phosphate groups attached to them. To prevent repulsion between these charged groups of adjacent lipopolysaccharide molecules, the LPS molecules bind strongly to divalent cations which are intercalated between these molecules (Nikaido, 2003). Chatterjee *et al*., (1976) showed that divalent cations such as Mg^{2+} ions played a crucial role in stabilising the outer membrane, therefore removal of these ions weakens the outer membrane resulting in leakage of periplasmic enzymes. Hardaway and Buller (1979) also confirmed the role of magnesium ions in stabilising the outer membrane. By treating cells with a metal chelator such as EDTA (Ethylenediaminetetraacetic acid) LPS molecules in the outer membrane were released from the outer membrane probably as a result of the removal of the Mg^{2+} ions by EDTA which in effect led to repulsion between LPS molecules and subsequent release from the outer membrane. Biosynthesis and transport of LPS from the cytoplasm, where it is made, to the outer

membrane is still not fully understood. A membrane transporter, MsbA, is believed to carry the newly synthesised LPS molecule across the inner membrane to the periplasm (Tran *et al*., 2008). Since the LPS core is hydrophobic, its transport across the periplasm is mediated by a chaperone. Recent studies have identified two proteins (LptA and LptB) which might play this role (Sperandeo *et al*., 2007). Both LptA and LptB are soluble proteins whose expression is under the control of σ^E (also known as σ^{24}). Transcriptional studies of *n*-butanol tolerant *E. coli* have identified *lptA* as being upregulated during *n*-butanol stress suggesting a possible contribution of the LPS to n-butanol tolerance (Reyes *et al*., 2011).

According to Nikaido and Vaara (1985), nearly half the mass of the outer membrane is protein. It has lipoproteins on the inner leaflet and integral proteins which span its length. The integral proteins are usually porins which act as a non-specific sieve that allows diffusion of nutrients and other compounds through the outer membrane. They also facilitate the efflux of waste from the cell to the outside environment. However, there are other outer membrane proteins that allow specific diffusion of compounds through the membrane. Neugebauer *et al*., (2005) confirmed previous studies which showed that the *E. coli* outer membrane protein, LamB allowed diffusion of maltodextrins across the outer membrane.

The outer membrane allows different compounds to pass through depending on the size and hydrophobicity of the compound. Even though it is permeable to hydrophilic solutes, it has a size exclusion limit and allows only hydrophilic solutes of size less than 600 Daltons to pass through (Nikaido and Vaara, 1985). In spite of the low specificity of the porins, the outer membrane is nearly impermeable to hydrophobic

compounds. Historically, Leive (1974) showed that the outer membrane resists the penetration of hydrophobic antibiotics, detergents and hydrophobic dyes. However, in a later publication, Black (1991) described a gene that encodes an outer membrane protein, FadL which facilitates the transport of hydrophobic long chain fatty acids across the outer membrane. The method of transporting these long chain fatty acids (LCFA) across the membrane has been described as lateral diffusion requiring no energy source and FadL works in concert with the inner membrane fatty acyl CoA synthase to transport the LCFA into the cytoplasm (van den Berg, 2010).

In between the inner and outer membranes of gram negative organisms is the periplasmic space filled with is a highly viscous matter (van Wielink *et al*., 1990) containing a peptidoglycan layer and a matrix of different biomolecules including solutes, proteins, oligo- and monosaccharides. The peptidoglycan layer is made up of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid linked together by a β-(1,4) glycosidic bond and helps to maintain the structural strength of the cell due to its rigidity (*Silhavy* et al., 2010).. The periplasmic proteins are soluble and play several important roles for the cell's maintenance and survival. One of the most important reactions that take place within the periplasm is the formation of disulphide bonds mainly catalysed by the protein DsbA in over 300 nascent proteins that are transported to the periplasm (McCarthy *et al*., 2000) since the periplasmic environment is highly oxidising. The formation of disulphide bonds in these proteins is crucial in stabilising their tertiary structure and maintaining them in the active form. Different protein chaperones that re-fold or degrade denatured proteins are also found in the periplasmic space as well as receptor proteins in two component systems that sense environmental signals.

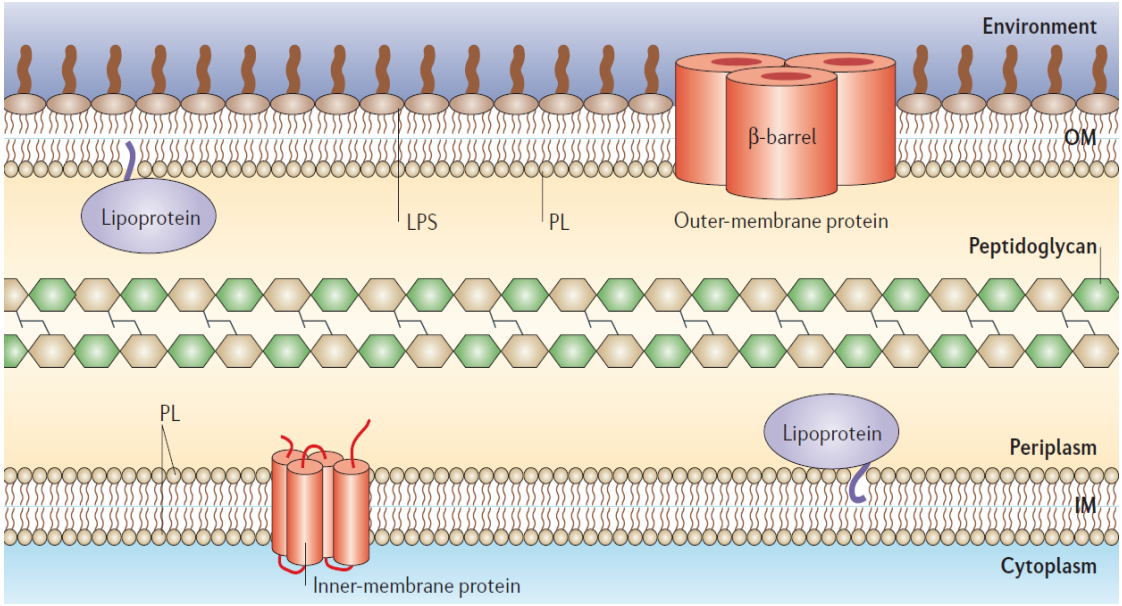


Figure 1.1: General structure of the *E. coli* cell envelope adapted from Ruiz *et al*., 2006

As the outer membrane is porous and allows transport of different chemicals into the periplasmic space, it is expected that sensitive structures in the periplasm such as proteins will be prone to damage from agents from the outer environment. However, Liu *et al*., (2004) challenged this notion and showed that periplasmic proteins resist aggregation when exposed to toxic environments implying that there is no need for molecular chaperones in the periplasm. Conversely, other studies (Denoncin *et al*, 2012; Xu *et al*., 2007) have shown the existence of key chaperones SurA and Skp in the periplasm which play roles in refolding and degrading proteins which have been denatured, possibly due to contact with harsh environments such as toxic compounds or heat.

The effect of harsh environments on the stability of the cell envelope has generated a lot of research interest, especially among groups aiming at increasing solvent tolerance in microbial hosts used to production of important chemicals in industry, such as biofuels. Several chemicals and compounds including detergents, metal

chelators, organic solvents, among others have been shown to have adverse effects on the membranes. Woldringh and van Iterson (1972) reported that anionic detergents such as sodium dodecyl sulphate (SDS) dissolve the plasma membrane of *E. coli* which results in the leakage of cytosolic biomolecules such as DNA, RNA and proteins. Using several techniques including atomic force microscopy, Alakomi *et al*., (2006) showed that metal chelators such as ethylenediaminetetraacetic acid (EDTA) displaced the lipopolysaccharide on the outer membrane and dissociated the membrane. EDTA acts by destabilising the outer membrane by removing the divalent cations that bridge the LPS. When these cations are removed, the LPS molecules repel each other and are then dissociated from the outer membrane. Organic solvents also disrupt the cell membrane. The toxicity of these compounds usually depends on the hydrophobicity and size of the compound (Sikemma *et al*., 1995). Hydrophobic compounds are usually more toxic since they accumulate in the phospholipid layer of the cell membrane.

1.5 Toxicity of organic solvents to bacteria

The first step in increasing tolerance is to understand why organic solvents are toxic. Toxicity of organic solvents has been attributed to different physical and chemical properties of solvents. Properties such as boiling point, solubility in water, viscosity and partition co-efficient in octanol-water (log P_{ow}) have been used to predict the toxicity of organic solvents (Sikkema *et al*. 1995). The octanol-water partition co-efficient is used to determine the hydrophobicity of organic compounds (Sikemma *et al*., 1995) and since hydrophobic compounds tend to accumulate more readily in the cell membrane which results in cell damage, there seems to be a correlation between solvent toxicity and the log P_{ow} of a compound.

A solvent's concentration in the membrane, generally based on its hydrophobicity, is important in determining how toxic it is and this usually depends on three factors – the concentration of the solvent in the water phase, its partitioning from the water phase into the membrane and the ratio of the volume of the two liquid phases (Neuman *et al*., 2005). One will expect hydrocarbons with very long carbon chains and a log P_{ow} value greater than 4 to be extremely toxic. On the contrary, these compounds with a log P_{ow} value greater than 4 are not toxic since their concentration in the water phase reduces drastically, and in effect, they are not able to attain a high membrane concentration. Therefore, solvents with log P_{ow} values between 1.5 and 4 are very toxic since such compounds partition well within the membrane due to their relative solubility in water (Neuman *et al*., 2005). The index value and index solvent are other ways of relating the solvent toxicity to microorganisms which further shows that microorganisms are able to tolerate a variety of solvents differently (Table 1.2). The index solvent refers to the most toxic solvent among the solvents that can be tolerated by a microorganism and the index value is the log P_{ow} of this most toxic solvent (Sardessai and Bhosle, 2002). This gives a little insight into the fact that microorganisms possess different survival strategies to withstand the effect of different solvents and it will be interesting to understand how different solvents affect the cell and the different stress responses that the cell puts up to protect itself.

Table 1.2: Levels of organic solvent tolerance found in different organisms

Solvent	Log P_{ow}	*Pseudomonas fluorescens* IFO3507	*Escherichia coli* IFO3806	*Bacillus subtilis* AHU1219	*Saccharomyces uvarum* ATCC26602
Dodecane	7.0	+	+	+	+
Decane	6.0	+	+	+	-
Nonane	5.5	+	+	+	-
n-Hexyl ether	5.1	+	+	+	-
Octane	4.9	+	+	+	-
Isooctane	4.8	+	+	-	-
Cyclooctane	4.5	+	+	-	-
Diphenyl ether	4.2	+	+	-	-
n-hexane	3.9	+	+	-	-
Propylbenzene	3.7	+	+	-	-
dichlorobenzene	3.5	+	-	-	-
Cyclohexane	3.4	+	-	-	-
Toluene	2.6	-	-	-	-
Benzene	2.1	-	-	-	-
n-butanol	0.88	-	-	-	-
Ethanol	-0.31	-	-	-	-
Index value		**3.4**	**3.7**	**4.9**	**7.0**

+, growth; -. no growth. Reference: Inoue and Horikoshi (1989).

1.6 Bacterial stress responses (Gram negative bacteria)

1.6.1 SOS response

Chemicals that cause DNA damage, arrest DNA synthesis or disrupt cell division, for example, 1-nitropyrene (Su *et al*., 1995), induce an SOS response in bacteria. Gram negative bacteria activate this response mechanism when its DNA is damaged. Gram positive bacteria, however, display different stress responses. For example, instead of an SOS response, *Streptococcus pneumonia* uses competence (Claverys *et al*., 2006). Key players in regulation of SOS response are the repressor, LexA and the inducer, RecA. In the absence of stress, there is usually an elevated amount of LexA within the cell with most of them bound to a 20-mer consensus sequence (5'-TACTG$(TA)_5$CAGTA-3') called SOS box in certain promoters (Fernández De Henestrosa *et al*., 2000). The binding of LexA to these promoter regions prevents the RNA polymerase from transcribing the SOS-regulated genes.

When the DNA is damaged by UV or by toxic chemicals, RecA binds to the single stranded DNA to form a nucleoprotein and acquires a co-protease activity which leads to the self-cleavage of LexA from the SOS box (Janion, 2008). The promoter of those SOS-regulated genes then become free and exposed to RNA polymerase which induces the expression of over forty genes that make up the SOS regulatory network (Butala *et al*., 2009). Even though most of the SOS-genes have not been assigned clearly defined functions (Fernández De Henestrosa *et al*., 2000), most of them are believed to play different roles in DNA repair.

1.6.2 General stress response

The general stress response is usually triggered in bacteria when they enter the stationary phase. High osmotic pressure, low pH, starvation and changes in temperature also induce this stress response (Foster, 2007). The key player of this response, RpoS (σ^s), binds to the RNA polymerase to direct the expression of several genes to counteract the effect of the stress. A genome-wide study revealed that genes involved in acid resistance, *gadABC*, were among the 481 genes induced by RpoS (Basak and Jiang, 2012). When cells are exposed to toxic concentration of butanol, they become unable to maintain their internal pH leading to a drop in the intracellular pH (Bowles and Ellefson, 1985), which can possibly induce a general stress response. The other genes induced by RpoS encode metabolic enzymes, transporters or are involved in stress management (Weber *et al*., 2005). Indeed, RpoS is able to induce spontaneous mutations by downregulating the expression of enzymes that are responsible for mismatch repair of DNA (Foster, 2007). When the general stress response becomes unneeded, the response mechanism is switched off by a non-coding RNA which degrades RpoS aided by a small protein, Hfq (Vogel *et al*., 2003).

1.6.3 Heat shock response

Most cellular proteins, depending on the organism, are sensitive to heat making it important to maintain the optimum temperature at all times to prevent denaturation. At elevated temperatures, the heat shock response mediated by σ^{32} or σ^{24} is activated. Sigma-32, a transcription factor encoded by *rpoH*, mainly upregulates heat shock proteins present in the cytoplasm whereas σ^{24} binds to RNA polymerase to specifically induce the expression of chaperones within the periplasm during envelope stress. Many of the heat shock proteins including GroEL, GroES, DnaK

and DnaJ are molecular chaperones. The heat shock proteins not only protect cells from heat shock but also provide some protection against high salt and heavy metals (Inbar and Ron, 1993). Also, genes encoding several heat shock proteins are upregulated during ethanol and butanol stress in *E. coli* and *Clostridium acetobutylicum* (Goodarzi *et al*., 2010; Reyes *et al*., 2011; Tomas *et al*., 2004).

In the presence of heat shock, the cell still manages to keep the transcription going by restoring denatured RNA polymerase. The molecular chaperone machineries, GroES/GroEL and DnaK/DnaJ/GrpE mediate this process by different mechanisms (Ziemienowicz *et al*., 1993).

The GroEL chaperone and its co-chaperonin, GroES, have been extensively studied (Hendrick and Hartl, 1993). GroEL is made up of fourteen subunits containing a hydrophobic apical domain which binds a protein substrate, an equatorial domain to which ATP binds and an intermediate domain (Motojima *et al*., 2000). In *E. coli*, the GroESL chaperone system binds to several soluble proteins which are unfolded and need refolding. Many of these substrates contain β-sheets with a hydrophobic core and their sizes usually range from 20 – 60 kDa (Desmond *et al*., 2004). In order to refold a denatured protein, GroES which is a heptameric molecule (Hunt *et al*., 1996) caps one end of *groEL* to form a grove in which the aggregated proteins are captured and refolded by interacting with the apical domain of GroEL. A conformational change results in the release of the refolded protein as a result of ATP binding to the GroESL complex (Wang and Chen, 2003).

The ClpB chaperone works with DnaK and its co-chaperones, DnaJ and GrpE, to restore unfolded proteins. When ClpB binds to aggregated proteins, ATP induces structural changes in ClpB which then exposes the aggregated proteins allowing

DnaK-DnaJ-GrpE to bind and refold the solubilised peptides into the native protein form (Goloubinoff *et al*., 1999). When the stress eases and the heat shock proteins sense a reduction in the amount of aggregated proteins, they self-regulate their own expression by repressing σ^{32} activity. GroESL inhibits transcription of σ^{32}-dependent genes by binding to σ^{32} to an extent similar to how DnaK/DnaJ binds to σ^{32} (Guisbert *et al*., 2004).

In thermophilic archaea, the DnaK/J-GrpE chaperone system is absent even though these organisms are able to survive in high temperature (Gribaldo *et al*., 1999). They possess a different class of chaperonins, generally referred to as the Group II chaperonins which interacts with a molecular chaperone known as Prefoldin encoded by the *phPFD* gene (Okochi *et al*., 2002). The chaperonin system in bacteria belongs to Group I. Prefoldin from the thermophile, *Pyrococcus horikoshii* OT3 is a hexameric protein with two α-subunits and four β-subunits and has been shown to increase tolerance to hexane (Okochi *et al*., 2008). It has six long coiled tentacles protruding from its barrel structure and giving the protein a jelly fish resemblance. The tentacles are joined to form a large cavity containing hydrophobic regions which capture non-native proteins (Ohtaki *et al*., 2008). The outside region of the tentacles binds to the substrate protein and delivers the protein to the chaperonin by interacting with the inner region of the apical domain of the latter (Martin-Benito *et al*., 2002).

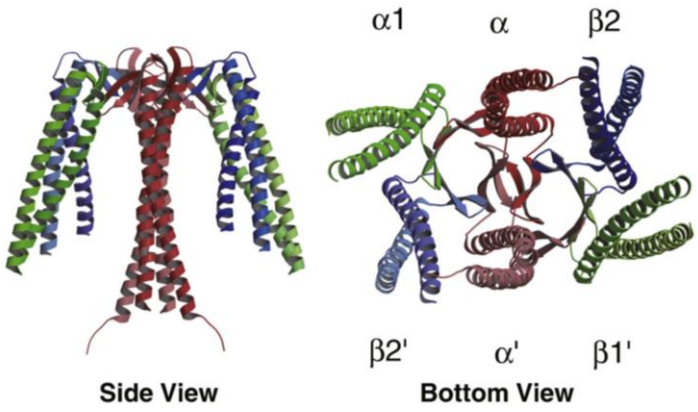


Figure 1.2: Structure of *Pyrococcus* Prefoldin adapted from Ohtaki *et al*., (2008)

The release of the substrate protein from Prefoldin to the chaperonin is facilitated by a specific interaction of the Prefoldin-substrate complex with the chaperonin (Zako *et al*., 2005). The fate of the aggregated protein in the chaperonin is still being debated. Contrary to a current model that suggests that ATP binding to the chaperonin causes a conformational change which forces the bound substrate to move and mechanically correct folding (Stuart *et al*., 2011), there is another proposition that suggests that ATP hydrolysis from the chaperonin releases the substrate into the inner grove of the chaperonin where refolding occurs (Douglas *et al*., 2011). The refolded protein is then released from the chaperonin.

1.6.4 Oxidative stress

In aerobic organisms, molecular oxygen found intracellularly is able to remove electrons from enzymes involved electron transfer, thereby producing partially reduced oxygen species in the form of H_2O_2, superoxide (O^{2-}) and hydroxyl free radicals (Imlay, 2008). These oxygen species attack different components of the cell including the membranes, DNA and proteins. Free radicals can attack the polyunsaturated fatty acids in the membrane and initiate lipid peroxidation, a

reaction that results in membrane disruption and loss of membrane fluidity. The sugar and nucleotide bases of DNA are also attacked, leading to single and double strand breaks within the DNA. Cellular proteins are also not spared and are oxidised by these reactive oxygen species (Cabiscol *et al*., 2000). The presence of organic solvents in the cell can lead to accumulated amounts of reactive oxygen species (ROS) beyond the cell's capability, resulting in an oxidative stress response. In bacteria, two main transcriptional regulators (SoxS and OxyR) are involved in the oxidative stress response.

1.6.4.1 The SoxRS regulon

In the presence of superoxide accumulation in the cell, a sensor protein, SoxR detects the stress and induces the expression of SoxS. SoxR is constitutively expressed and binds to the SoxS promoter with a reduced form of its iron-sulphur ($[2Fe\text{-}2S]^{2+}$) cluster in the absence of superoxide anions. In the presence of superoxide anions, the iron-sulphur cluster of the bound SoxR becomes oxidised altering binding to the SoxS promoter which leads to transcription of SoxS (Pomposiello and Demple, 2001). SoxS then drives the expression of several genes including *sodA*, *acrAB* (responsible for drug efflux), *tolC* (outer membrane component of a drug efflux pump), among others (Imlay *et al*., 2008) and has been shown to increase tolerance to organic solvents, suggesting a link between oxidative stress and solvent toxicity (Nakajima *et al*., 1995). The *sodA* gene encodes a manganese-containing superoxide dismutase which scavenges the superoxide anion and detoxifies it by catalysing the dismutation of the anion into hydrogen peroxide and molecular oxygen (Luke *et al*., 2002). Expression of *acrAB* is also induced by SoxS during oxidative stress and has been shown to be upregulated when cells are exposed to organic solvents (White *et*

al., 1997). The biological function of the former will be discussed later in this review.

1.6.4.2 OxyR transcriptional regulator

To eliminate intracellular peroxide, the OxyR transcriptional activator binds to regions near or overlapping the promoter of at least 20 genes including *katG*, *ahpC*, *dps*, *gorA* among others (Pomposiello and Demple, 2001). The *katG* gene encodes a catalase which catalyses the conversion of hydrogen peroxide to water and oxygen. Also functioning as a scavenger of intracellular hydrogen peroxide is the alkyl hydroperoxidase encoded by *ahpC* (Guimaraes *et al*., 2005). During the stationary phase of bacterial cell growth or during starvation, a DNA-binding protein (Dps) is expressed which is encoded by the *dps* gene. This gene also plays a role in oxidative stress response and is regulated by the OxyR regulon. Dps binds to cellular DNA and protects it from the damaging effects of hydroxyl radicals generated by a reaction (Fenton Reaction) between ferrous iron (Fe^{2+}) and hydrogen peroxide. It can also remove Fe^{2+} ions by binding them and preventing the formation of toxic hydroxyl free radicals from being generated from a Fenton reaction (Calhoun and Kwon, 2010; Haikarinen and Papageorgiou, 2010). Even though the contribution of *dps* to acid resistance in bacteria has been established (Choi *et al*., 2000) no work has been done to investigate the protective effect of *dps* on cells undergoing solvent-induced oxidative stress.

In the presence of hydrogen peroxide, OxyR becomes oxidised at Cys^{199} and Cys^{208} which forms a stable disulphide bond (Zheng *et al*., 1998). Oxidised OxyR then binds to the promoters of the OxyR-induced genes where induction of these genes is initiated by the interaction of the oxidised OxyR with the carboxyl terminal of the α-subunit of RNA polymerase (Pomposiello and Demple, 2001). In the absence of

stress, however, OxyR autoregulates its own inactivation by inducing the expression of a glutathione synthetase (GshA), glutaredoxin A (GrxA) and glutathione reductase (GorA) which work together to reduce the disulphide bond between Cys^{199} and Cys^{208} of OxyR (Aslund *et al*., 1999; Zheng *et al*., 1998). The reduction of this disulphide bond inactivates OxyR (Imlay, 2008).

1.7 Ethanol stress responses and tolerance

Very early reports on work done to elucidate the effect of ethanol on bacterial cells show that ethanol alters the aqueous environment of the periplasm and inhibits several membrane-bound enzymes. Since key enzymes inhibited are those involved in peptidoglycan biosynthesis, inhibition of these enzymes prevents cross-linking of the peptidoglycan and results in cell lysis (Ingram and Vreeland, 1980). Further work done by Dombek and Ingram (1984) showed that ethanol interacts with the lipid bilayers of membranes and in effect, reduce the fluidity of the plasma membrane. It was also observed from that work that cells exposed to ethanol had a reduced lipid/protein ratio in response to the changes in fluidity of the cell membrane. This work and other studies (Berger *et al*., 1980; Buttke and Ingram, 1980) have confirmed the effect of alcohols such as ethanol on the cell membrane. These findings are not surprising as the cell membrane is the primary point of contact of any chemical entering the cell and it is expected that the cell will respond accordingly to protect itself from the toxicity of any compound working its way into the intracellular space. The effect of ethanol on enzymes involved in glycolysis and fermentation has also been studied. Millar *et al*., (1982) reported that pyruvate decarboxylate, an enzyme involved in fermentation is significantly inhibited by 5%

w/v ethanol leading to cessation of ethanol production in yeast and *Zymomonas mobilis*. Glycolytic enzymes were more stable and 50% inhibition of these enzymes occurred only when intracellular ethanol concentrations were above 12-15% w/v in the medium. Above this concentration of ethanol, fermentation ceased.

It is evident that ethanol has several effects on the bacterial cells. A prudent way of improving tolerance to ethanol stress will be to examine how the cell naturally responds to this stress in order to enable us understand what mechanisms might be important in increasing ethanol tolerance. Knowledge of these mechanisms will be important for engineering tolerance. Apart from membrane modification which had been previously reported by Ingram and Dombek (1989), the first systems approach to discover other possible mechanisms of tolerance was observed when the gene expression levels of laboratory-evolved *E. coli* strains were compared to that of the parent strain (Gonzalez *et al*., 2003). It was found from this work that a large fraction of genes with different expression levels were globally regulated. Genes involved in glycine and betaine metabolism as well as those involved serine uptake (*sdaC*) and deamination (*sdaB*) were upregulated during ethanol stress. The role of these factors in tolerance to ethanol was confirmed by supplementing the media with serine and betaine (Gonzalez *et al*., 2003).

Further advances in the development of genetic tools have made it possible to identify a whole myriad of other genes that respond to ethanol stress. Also, with genome sequencing getting relatively more affordable and the emergence of novel molecular techniques such as Scalar Analysis of Library Enrichments referred to as SCALEs (Struble and Gill, 2009), recent work has taken advantage of these technologies to further elucidate genome-wide response to ethanol stress (Woodruff

et al., 2013a). Induction of genes involved in the biosynthesis of some amino acids has been reported to increase ethanol tolerance. Notable among these upregulated amino acid biosynthesis genes is *serA* which encodes α-ketoglutarate reductase, the enzyme that catalyses a committed step in serine biosynthesis (Woodruff *et al*., 2013a). In a related study in which lab-evolved ethanol tolerant *E. coli* strains were characterised, genes involved in the biosynthesis of arginine, histidine and branched-chain amino acids such as valine, leucine and isoleucine were significantly upregulated in ethanol tolerant strains (Horinouchi *et al*., 2010). Apart from these, Gonzalez *et al*., (2003) also reported that supplementing liquid cultures with serine enhances tolerance to ethanol. It might be interesting to investigate why these particular amino acids and not others increase ethanol tolerance.

The ethanol tolerance phenotype is multifaceted and it appears several mechanisms interact or work independently to increase tolerance. Genes involved in galactitol metabolism (*gatB, gatC, gatY, gatZ*), phosphate transport (*phoB, phoU, pstA, pstB, pstC, pstS*), mannose uptake (*manXYZ*) and heat shock response have been shown to be upregulated during ethanol stress response (Horinouchi *et al*., 2010). Some of these such as *manXYZ* have been reported to increase tolerance to other organic solvents such as n-hexane, toluene, p-xylene and cyclohexane (Okochi *et al*., 2007). Other genome-wide studies have demonstrated that peptidoglycan biosynthesis and metabolic rewiring are important responses to ethanol stress (Nicolaou *et al*., 2012; Goodarzi *et al*., 2010). This could be because the cell will want to fortify itself and limit the amount of ethanol entering it and should ethanol get into the cell, rewiring its metabolism to convert the solvent to acetylCoA which is fed into the TCA cycle will be an appropriate way to detoxify it.

Several other genes and mechanisms involved in ethanol stress response and tolerance such as LPS biosynthesis, fatty acid oxidation, translation, to name a few have been reported (Woodruff *et al*., 2013a). While it is possible to suggest a possible contribution of some of these genes to ethanol tolerance, the role of many of the genes cannot be predicted and indeed, the involvement of other genes in ethanol tolerance is still being debated. One such mechanism, whose role in ethanol tolerance is being debated (as a result of different studies reporting conflicting results) is the acid stress response. The *gad* genes and genes regulated by the PhoR/PhoB two-component regulatory system which are known to be upregulated during acid stress, have also been shown to respond to ethanol stress (Horinochi *et al*., 2010) signifying that ethanol elicits stress responses similar to acid stress. However, another study has shown that overexpressing a different set of genes belonging to the acid tolerance pathway made cells more sensitive to ethanol stress (Goodarzi *et al*., 2010). The type of growth media used for these experiments can affect the results obtained and this could be a possible explanation for these inconsistent results. This also highlights the need to screen these stress response genes individually against ethanol and other organic solvents under different conditions in order to gain some reliable understanding of their possible role in tolerance. Therefore, identifying a set of tolerance modules will pave the way to further increase tolerance by using a combinatorial approach.

The possible role of genes involved in glycine-betaine metabolism was confirmed in another study by screening overexpressing and transposon insertion libraries (Goodarzi *et al*., 2010). The involvement of osmolytes like glycine and betaine in *E. coli* ethanol tolerance suggests a possible overlap in osmotic stress responses and ethanol stress response. Also, overexpressing *otsA* and *otsB* which are involved in

trehalose biosynthesis, increases tolerance not only to osmotic shock but to ethanol as well (Purvis *et al*., 2005; Woodruff *et al*., 2013b). On the contrary, a genome-wide screen of genes involved in different stress responses in *Saccharomyces cerevisiae* showed no possible overlap between ethanol stress response genes and osmotic shock response genes (Auesukaree *et al*., 2009). This further adds to the complexity of ethanol tolerance and also shows how different organisms respond differently to ethanol stress.

Over-expression or deletion of single genes in *E. coli* has not been able to significantly increase tolerance to organic solvents since solvent tolerance is a phenotype controlled by many genes. The association of global gene regulators in ethanol tolerance confirms the involvement of a multitude of genes in the cell's attempt to deal with ethanol toxicity. Therefore, investigation of the role of global regulators in ethanol stress response is a step in the right direction towards increasing tolerance to ethanol. However, overexpressing a global regulator, Fnr, rather decreased ethanol tolerance in a strain of *E. coli* (Gonzalez *et al*., 2003). Still focussing on the possible advantage of exploiting the cell's global transcription machinery to increase tolerance, a different approach was used to increase ethanol tolerance by mutating the *rpoD* gene which encodes the main sigma factor, σ^{70} in *E. coli*. Sigma factors recruit the RNA polymerase to specifically bind to a promoter to initiate transcription. By using mutant forms of σ^{70}, certain genes were differentially expressed leading to an increase in ethanol tolerance in *E. coli* (Alper and Stephanopoulos, 2007). A similar approach where the yeast transcription factor, Spt15p, was mutated to control transcription of several genes had been used previously to increase ethanol tolerance in yeast (Alper *et al*., 2006). The inability of Fnr but ability of RpoD to increase ethanol tolerance means that in order to increase

tolerance by using the cell's global transcriptional machinery, it is important to target the right global regulators. Another lesson from this work is that by modulating the expression of the right set(s) of genes, tolerance can be enhanced.

1.8 Mechanisms of butanol toxicity

Similar to the approaches taken to investigate ethanol stress response and tolerance, work done to increase butanol tolerance has involved identifying key genes and mechanisms involved in butanol stress response. This knowledge can be used to reconstruct tolerance pathways. Studies investigating butanol stress response have received very limited attention until recently.

Selecting the right mechanism of tolerance is important for generating butanol-tolerant hosts. The first step in this endeavour is to identify such mechanisms that exist naturally to make bacteria tolerant to butanol. Brynildsen and Liao (2009) first did this by using a systems biology approach studying gene expression, transcriptome analysis and network component analysis to map genes that respond to isobutanol stress in *E. coli*. In that report, *arcA* was shown to be activated as a result of quinone malfunction which was possibly as a result of a disruption in the membrane-quinone interaction. This suggests an element of membrane damage when cells are exposed to isobutanol. Thus, enzymes that require quinones for their activity will be affected. During respiration in *E. coli*, ubiquinone act as one of the components in a chain of electron transporters. It is a freely mobile hydrophobic molecule located within the lipid bilayer of the membrane which can accept one or two electrons at a time and pass them on to the cytochrome b-c_1 complex while pumping out protons (Alberts *et al*., 2002). The proton gradient generated, enables

ATP synthase to make ATP. In a final step step, the cytochrome b-c_1 complex passes on the electrons to oxygen (Alberts *et al*., 2002). Since quinone activity is membrane related and also plays a role in respiration, it implies that respiration will be impaired as well. Transcriptional factors, *phoB* and Fur, are also affected as result of quinone inhibition. Apart from quinone inhibition, several enzymes involved in branched chain amino acid synthesis are repressed when *E. coli* is exposed to isobutanol.

A mutant *E. coli* strain isolated from an adaptive lab evolution experiment was sequenced to identify important mutations that had increased resistance of this strain to isobutanol (Atsumi *et al*., 2010). Four genes were inactivated in the resistant strain by the insertion of transposable elements. These genes were *tnaA, gatY, acrA* and *yhbJ*. A fifth, the *marCRAB* operon was entirely deleted in this strain. The gene, *yhbJ* negatively regulates the expression of *glmZ. glmZ* encodes an ezyme involved in the biosynthesis of N-acetylglucosamine, which is used for peptidoglycan and LPS synthesis. Mutations in *marC, acrAB* and *gatYABCD* have also been reported (Minty *et al*., 2011). The mutant strain showed tolerance to *n*-butanol as well, but not to ethanol suggesting that the mechanism of tolerance of *E. coli* to isobutanol is similar to other longer chain alcohols.

These reports show that the effect of isobutanol is primarily on the cell membrane and the cell responds to it by synthesising components of the membrane. Using these novel transcriptional studies to investigate isobutanol tolerance paved the way for investigation of other mechanisms of toxicity that exist when cells are exposed to *n*-butanol. Several biological processes and a variety of associated genes have been shown to be either upregulated or downregulated when *E. coli* is exposed to *n*-butanol. Like the isobutanol stress response, genes involved in the production of

membrane lipoproteins, fatty acid metabolism, lipopolysaccharide (LPS) synthesis and peptidoglycan biosynthesis are upregulated suggesting an effect on the membrane (Reyes *et al*., 2011; Reyes *et al*., 2012; Rutherford *et al*., 2010; Seregina *et al*., 2012). Genes involved in ferrous iron transport (*feoA* and *entC*), efflux pumps (*argO*), amino acid transporters and others that indicate oxidative stress (*ompT*, *yjaA* and *yodD*) appear to be unique to *n*-butanol stress (Reyes *et al*., 2011; Reyes *et al*., 2012). Very little is known about the role of ferrous iron metabolism during the cell's adaptation to *n*-butanol and this mechanism is intriguing since increased intracellular amounts of ferrous iron is often detrimental and causes oxidative stress in bacteria (Sikora *et al*., 2009). Confirming the role of ferrous iron in butanol tolerance, Lee *et al*., (2011) reported that supplementing the culture media with ferrous iron increased *E. coli* tolerance to butanol. FeoA is a small hydrophilic protein (8.4 kDa) found in the cytosol of bacteria which mediates the uptake of ferrous iron by binding to a ferrous iron transporter, FeoB (Cartron *et al*., 2006; Kim *et al*., 2012). Its contribution to butanol tolerance is indeed interesting and requires further investigation.

Unlike isobutanol stress response, the upregulation of *acrAB* has been established in *n*-butanol response. There is also evidence suggesting that *marA* is highly upregulated during 1-butanol stress (Zhang *et al*., 2012). During isobutanol exposure, deleting the *acrA* gene increased tolerance (Atsumi *et al*., 2010; Minty *et al*., 2011) meaning that the AcrAB-TolC system is not required for the extrusion of isobutanol even though Rutherford *et al*., (2010) have showed that *n*-butanol upregulates this system in *E. coli*. It was not surprising that *marCRAB* was also inactivated in the isobutanol tolerant strain since *marA* present in that operon induces the expression of the *acrAB-TolC* system. This suggests that even among the butanols, *E. coli* displays

different stress responses which may be explained by the slight differences in the chemical structures. It also shows the specificity of these efflux pumps to different organic solvents which should be considered during rational engineering of tolerant strains. On the other hand, AcrAB-TolC might not actually increase *n*-butanol tolerance as was observed when this pump was screened against *n*-butanol for tolerance (Dunlop *et al*., 2011) supporting the claim that if a gene is upregulated it does not necessarily increase tolerance. The AcrAB-TolC efflux system is one of the most dominant pumps in *E. coli.* According to Higgins *et al*., (2004) the main transporter of this efflux system is the inner membrane component, AcrB. This is a trimer with a transmembrane domain having 12 membrane-spanning alpha helices and a large periplasmic domain. AcrB is linked to the outer membrane protein, TolC by the membrane fusion protein which spans the entire periplasmic space, AcrA. AcrAB-TolC has been shown to confer solvent tolerance and to oxidative stress suggesting an overlap of these two stress responses (White *et al*. 1997; Nikaido and Takatsuka, 2009). MarA, one of the proteins encoded by the marCRAB operon, differentially affects the expression of about 62 genes (Barbosa and Levy, 2000) including *acrAB* confirming its contribution to antibiotic and solvent tolerance by pumping toxic chemicals out of the cell. It also bears at least 45% homology with SoxS and upregulates most of the SoxS-genes including *sodA* which shows that the mar (multiple antibiotic resistance) phenotype is related to superoxide stress response (Martin *et al*., 1996).

1.9 Engineering *n*-butanol tolerance

Butanol stress response is indeed a very complicated process involving many genes and mechanisms as shown in the previous section. Increasing tolerance to organic solvents such as *n*-butanol requires the use of different strategies. All the approaches that have been employed to increase tolerance to butanol can be categorised into two groups – random engineering and rational engineering. With random engineering, solvent tolerant strains are made by the introduction of random and non-specific mutations within the genome of the bacterium whereas the rational engineering approach involves deliberately engineering bacteria with particular genes to achieve a desired purpose.

Random engineering is used to obtain butanol tolerant strains usually done by treating the cells with mutagens such as UV or *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine followed by screening of mutants in toxic concentrations of the organic solvent (Hermann *et al*., 1985). *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine acts as a mutagen by methylating the nucleotides, cytosine, adenine and guanine to form 3-methylcytosine, 1,3-methyladenine and 7-methylguanine respectively (Sussmuth *et al*., 1972). These methylated bases can be removed from the DNA, thus creating gaps that may be filled with incorrect bases during DNA repair. Mispairing of bases also occurs during replication when this methylated DNA is used as a template. Others have also used this approach to obtain solvent tolerant mutants by using error-prone PCR to introduce random mutations in specific genes (usually transcription factors) of bacteria. *E. coli* was made tolerant to 0.5% *n*-butanol by introducing random mutations in its *rpoA* gene which encodes the alpha-subunit of the RNA polymerase involved in transcription (Klein-Marcuschamer *et al*., 2009). Sequencing the mutant *rpoA* of this tolerant strain revealed that a mutation resulting in the insertion of a stop

codon in the alpha subunit of the RNA polymerase led to the expression of a truncated protein. Membrane damage was limited in this mutant strain of *E. coli* explaining its tolerance to *n*-butanol. Again, by introducing mutations into an artificial transcription factor made up of a zinc finger binding protein and a cyclic AMP receptor protein, an *E. coli* strain which was tolerance to 1.5% butanol was isolated. Furthermore, manipulating the expression of several genes by mutating the global transcription factor cyclic AMP receptor protein (CRP) can result in increasing tolerance to butanol in *E. coli* (Zhang *et al*., 2012). Not only does CRP activate the transcription of several genes involved in catabolism it also negatively regulates transcription at a number of promoters (Botsford and Harman, 1992; Khankal *et al*., 2009). This mutation results in the high expression of several genes including the multiple antibiotic resistance gene, *marA* which implies that the activation of other mechanisms such as AcrAB-TolC to extrude butanol from the cell is important in developing tolerant strains. All of these results show that controlling the expression of a wide variety of genes though the modification of transcription factors is a possible way of increasing butanol tolerance. Bacteria use efflux pumps to extrude toxic chemicals from the cells. Therefore, engineering *E .coli* with such efflux pumps will be useful in getting rid of intracellular amounts of butanol especially from *E. coli* engineered to produce butanol. Fisher *et al*., (2013) used an error-prone PCR method to generate mutant forms of one of the components of the native *E. coli* efflux pump, AcrB which was able to increase *n*-butanol tolerance by pumping out intracellular *n*-butanol even though the AcrAB efflux system would normally not pump out *n*-butanol (Dunlop *et al*., 2011). The downside to using mutant versions of genes to increase tolerance is that there is often competition between the mutant and wild type form of the transcriptional activators making it

difficult to determine the exact effect of the mutant form alone. However, this can be overcome by using genome editing strategies to replace the wild version with the mutant version.

Another random engineering approach is the use of lab-based evolution which involves a long term growth of cells in toxic concentrations of butanol followed by the selection of resistant mutants. By using this approach it has been possible to obtain *E. coli* strains that can tolerate up to 1.25% *n*-butanol (Seregina *et al*., 2012). Butanol tolerance was increased even further when the important mutations obtained from a lab-based experiment were combined by genome shuffling. Tolerant strains were able to survive a short term exposure (ie 1 hour) to 2% *n*-butanol (Reyes *et al*., 2012). Even though this seems promising, the possibility of further increasing butanol tolerance in *E. coli* should be iinvestigated.

Recent efforts in increasing butanol tolerance in *E. coli* have employed a more rational approach. Based on available evidence that several heat shock proteins which serve as a general stress response system in E. coli are upregulated during butanol stress (Brynildsen *et al*., 2009; Reyes *et al*., 2011), butanol tolerance was achieved by overexpressing several heat shock proteins in *E. coli*. However, the tolerant strains were only able to survive up to 1% *n*-butanol (Zingaro and Papoutsakis, 2012; Zingaro and Papoutsakis, 2013). Engineering *E. coli* to express metallothioneins increases tolerance to *n*-butanol. Reactive oxygen species produced intracellularly during *n*-butanol stress (Rutherford *et al*., 2010) can damage several components of the cell including the cell membrane, protein and DNA. Metallothioneins are cysteine-rich proteins found in animals, plants and fungi. These proteins are metal binding and have also been shown to trap harmful oxidants such as

hydroxyl radicals and superoxides. Very recently, Chin *et al*., (2013) demonstrated that expressing a metallothionein from tilapia on the cell membrane of *E. coli* by fusing it to an outer membrane protein, OmpC, increased *E. coli* tolerance to 1.5% *n*-butanol possibly by reducing oxidative stress induced by the solvent.

Current knowledge obtained from transcriptional and proteomic analysis has provided guidance in rational engineering of solvent tolerance microbes. Overexpressing a few genes upregulated during *n*-butanol stress such as *feoA* and *entC* individually increases tolerance to *n*-butanol (Reyes *et al*., 2011). Also deleting genes which are downregulated in the presence of *n*-butanol has been shown to increase tolerance to the solvent (Atsumi *et al*., 2010; Reyes *et al*., 2011). There are still a lot of genes differentially expressed during cell stress and these should be screened for tolerance against butanol since the fact that a gene is upregulated does not necessarily mean it increases tolerance to butanol. At the moment, the most butanol tolerant *E. coli* strain developed by either the random engineering approach or the rational engineering approach can tolerate 2% *n*-butanol only for a few hours which is not impressive if *E. coli* will be used as a host for producing biobutanol. There is the need to further increase tolerance of this host to more appreciable level. A good level of tolerance should go beyond 2% until the limit of tolerance is achieved. These results show that overexpressing or deleting single genes do not increase *n*-butanol tolerance therefore, combining several genes might be able to aid rational engineering of more robust hosts. There is limited information available in literature showing the use of a combinatorial strategy to obtain extremely resistant bacteria.

1.10 Toxicity of fermentation inhibitors

To convert lignocellulosic materials to biofuels, the biomass is first pre-treated to expose the cellulose and hemicellulose for enzymatic conversion into simple sugars which can then be fermented to produce fuels. Pre-treatment of lignocellulose usually involves hydrolysis with acid at high temperatures (Grethlein and Converse, 1991). Under high temperature and pressure, xylose produced from hemicellulose breakdown is converted to furfural whereas, the hexose sugars obtained from cellulose hydrolysis are converted to 5-hydroxymethyl furfural (Ulbricht *et al*., 1984). Also, not only are organic acids such as levulinic acid and formic acid formed from the breakdown of furfural and 5-methylhydroxy furfural, but partial degradation of lignin produces phenolic acids (Bardet *et al*., 1985; Ulbricht *et al*., 1984). All of these by-products are toxic to the microbial hosts and affect their productivity in different ways by inhibiting growth and fermentation (Modig *et al*., 2002). Furfurals have also been shown to inhibit hexokinase in yeasts (Banajeree *et al*., 1981).

Several strategies such as engineering detoxification pathways in microbial hosts have been used to eliminate furfural and 5-methyl furfural toxicity in *E. coli* and yeasts (Liu *et al*., 2009). *E. coli* cells try to detoxify furfural and 5-hydroxymethyl furfural by converting these inhibitors into a less toxic alcohol in a NADPH-intensive reaction catalysed by a NADPH oxidoreductase, YqhD (Miller *et al*., 2009a). In a related work, transcriptional analysis of furfural-exposed cells show that when cells are exposed to furfural, genes involved in cysteine and methionine biosynthesis are upregulated (Miller *et al*., 2009b). These two reports suggest a possible link between sulphur assimilation and NADPH depletion(probably by cysteine and methionine biosynthesis) during furfural challenge. Depletion of the cell's NADPH reserves as a result of sulphur assimilation is one of the mechanisms

that make furfural toxic to *E. coli* (Miller *et al*., 2009b). By deleting *yqhD* and overexpressing a combination of a NADH-dependent oxidoreductases, *fucO* and *ucpA*, furfural tolerance was achieved (Wang *et al*., 2013). It will be interesting to screen a wide range of stress response genes against these fermentation inhibitors. By doing so, it will be possible to generate tolerance modules suited for these inhibitors and this will also increase our knowledge of other mechanisms of toxicity that might be taking place when cells are grown in the presence of these inhibitors.

1.11 Membrane modification by *cis-trans* isomerisation

Current interest in using microorganisms to produce bioethanol from cellulosic materials has led to a number of studies looking at the effect of ethanol on the cell membrane. Ethanol accumulates in the lipid bilayer and alters the membrane fluidity. Dombek and Ingram (1984) reported that when *E. coli* plasma membranes are exposed to ethanol, the membranes become rigid and proposed a model which states that the hydroxyl groups of alcohols interact with the hydrophilic groups at the surface of the membrane bilayer while the hydrocarbon chain of the alcohol penetrates the core of the bilayer. This implies that short chain alcohols will only be interacting close to the membrane surface while longer chain alcohols would have a more profound effect on the lipid-lipid interactions in the hydrophobic core of the bilayer therefore making longer chain alcohols more toxic. Butanol, a more preferred biofuel (in comparison to ethanol) is even more toxic than ethanol since it has a longer carbon chain and is more hydrophobic than the latter. Huffer *et al*., (2011) reported that butanol increases membrane fluidity which makes the cell prone to cofactor leakage. Their work showed that *E. coli* and other microorganisms increase the saturation of fatty acids in the membrane to counteract the fluidising effect of

butanol on the membrane. Griepernau *et al*., (2007) confirmed these observations and showed in their work that alcohols accumulate in the lipid bilayer by interacting with the lipids in the bilayer with their hydrocarbon chain. It was also reported from this same work that longer chain alcohols increase the thickness of the bilayer. Both models can potentially increase butanol tolerance. It will be interesting to investigate these in parallel in order to determine which membrane modification approach is more relevant in increasing butanol tolerance.

In the presence of organic solvents, microorganisms adjust the membrane fluidity to protect the membrane from damage. This is brought about mainly by changes in the fatty acid composition of membrane lipids by increasing the ratio of saturated fatty acids to unsaturated fatty acids, increasing chain length of the membrane fatty acids and by converting unsaturated fatty acids to cyclopropane fatty acids (Hazel and Williams, 1990; Zhao *et al*. 2003). In *Pseudomonas,* a bacterium known to be resistant to organic solvents, a different kind of membrane modification takes place in the presence of organic solvents where the *cis*-unsaturated fatty acids are converted to the *trans* isomer (Heipieper *et al*., 2003). Tolerance of *Pseudomonas* to phenols and other solvents is as a result of this *cis-trans* conversion and it has been shown that the increase in *trans*-fatty acids was accompanied by a decrease in the amount of *cis*-fatty acids, while the total amount of both remained constant at any concentration of added solvent. The conversion is a single step isomerisation reaction that does not require any energy input due to the negative free energy nature of the reaction (Heipieper *et al*. 1992). The key player in this reaction is the *cis-trans* isomerase (CTI) which has been well-studied and discussed by Heipieper *et al*. (2003). The *cti* gene has been found only in some *Pseudomonas* and *Vibrio* species

even though gene sequences which resemble that of *cti* have been found in the genomes of bacteria belonging to the genera *Methylococcus* and *Nitrosomonas* (von Wallbrun *et al*. 2003). CTI is charge-neutral protein with a size of 80 kDa possessing an N-terminal hydrophobic signal sequence which is cleaved off after targeting the enzyme to the periplasmic space (Pedrotta and Witholt 1999). CTI was purified by Pedrotta and Witholt (1999) from the periplasmic fraction of *Pseudomonas oleovorans* and its mechanism of reaction was well illustrated by von Wallbrun *et al*. (2003). Firstly, there is the formation of an enzyme-substrate complex followed by an abstraction of an electron from the *cis*-double bond by Fe^{3+} ions required by the enzyme. The double bond is then reconstituted and transformed into the *trans*-configuration. Even though CTI is a cytochrome c-type protein that bears a haem group, its activity does not depend on NADPH, ATP or oxygen (Holtwick *et al*., 1999). CTI is constitutively expressed. However, its activity depends on the presence of organic solvents and toxic ions. When this observation was first made, it was quite baffling, but later work showed that toxic levels of metals and organic solvents induce the *cis-trans* isomerisation activity of the enzyme. Explaining this, Pedrotta and Witholt (1999) hypothesised that since CTI only uses free fatty acids as a substrate, an enzyme triggered by organic solvents known as phospholipase, might be induced in the presence of organic solvents which will lead to cleavage of free fatty acids from the phospholipids to be converted to the *trans* form by CTI. It should also be noted that the *cis-trans* isomerisation mechanism, is just a short term response in *Pseudomonas* while it prepares other tolerance strategies to fight the stress intracellularly (Keweloh and Heipieper, 1996). The survival rate of a *cti* knockout mutant of *Pseudomonas putida* was lowered when challenged with toluene (Junker and Ramos, 1999) which further confirmed the involvement of *cti* in solvent

tolerance. It will be interesting to investigate the effect of overexpressing *cti* in *E. coli* tolerance to *n*-butanol since this has not been previously investigated.

1.12 Solvent Tolerance versus Gas stripping

Quite recently, a technique which involves sparging gas through a fermenter to remove volatile organic compounds (butanol, for example) which can be recovered by condensation has been used in downstream processes to remove liquid fuels from bioreactors. The technique, known as gas stripping, has been shown to improve the yield of the final products of organic solvents by preventing their inhibitory action on the microbial hosts used for the bioconversion (Ezeji *et al*., 2005). By applying gas stripping, a record high concentration of 30 g/l 1-butanol was produced in bioreactor made up of *E. coli* engineered to produce 1-butanol (Shen *et al*., 2011).

These advantages of gas stripping undermine the need to develop solvent tolerant microbial hosts since product toxicity is eliminated by the removal of the organic solvent. However, this technique might come with some extra expenditure such as the cost of the setup, gases used and the energy involved in the process. In an ideal process to convert biomass to biofuels in consolidated bioprocessing, not only should the final biofuel product be removed but also the fermentation inhibitors that are produced from the degradation of the biomass. This will definitely come with an extra cost and make the process more expensive. All of the published reports demonstrating gas stripping as a superior technique to increase yield by reducing product toxicity showed the conversion of sugars to fuel. None has shown the removal of fermentation inhibitors produced in the initial step of biomass degradation in the sugars. The economics involved in gas stripping is yet to be worked out and there is the need to compare that with the advantages of using robust

microbial hosts. Again, since the mechanisms of organic solvent tolerance overlap with other stresses (as shown in this review), such tolerant microbes can be used for different purposes including production of industrially relevant chemicals that cannot be normally removed by gas stripping. Furthermore, solvent tolerant hosts will find other use in bioremediation. Therefore, the need to investigate solvent toxicity in bacteria with the aim of improving tolerance remains valid and with the emergence of novel synthetic biology tools and the concept of standardisation this can be achieved.

1.13 Application of synthetic biology

An emerging field of biology, synthetic biology seems to be the way forward in modern genetic engineering. Synthetic biology has been defined as 'the engineering of biology: the synthesis of complex, biologically based (or inspired) systems, which display functions that do not exist in nature'' (Serrano, 2007). This involves the use of engineering principles to build living systems. This means that novel biological systems and circuits can now be composed from scratch by designing, synthesising and assembling pieces of DNA with specific functions. Unlike the conventional method of genetic engineering that deals with altering some components of biological systems, synthetic biology has the advantage of enabling scientists build complex biological systems from standard parts which can be transferred into a biological host.

Synthetic biology relies on the use of standard parts that have been well characterised and this resulted in the setting up of a repository of parts such as the MIT's Registry of Biological Parts (http://parts.mit.edu/). These standard DNA parts called BioBrick parts are flanked by *EcoRI* and *XbaI* restriction sites upstream at the 5' end while

downstream at the 3' end, *SpeI* and *PstI* sites are situated (Knight, 2003). It is important that none of these restriction sites should be found anywhere else in the DNA part and any such sites should be removed by point mutations if found. These restriction sites placed upstream and downstream of the BioBrick part give it the flexibility required for combining any two different parts. The *XbaI* and *SpeI* recognition sequences (TCTAGA and ACTAGT respectively) form compatible overhangs allowing two different parts to be ligated at this point (Knight, 2003). This ligation forms a 'scar' sequence which can neither be recognised nor cut by the *XbaI* or *SpeI* restriction enzyme (Figure 1.3). By using the appropriate restriction enzymes it is possible to place one BioBrick part either upstream or downstream of an existing one to form a new part bearing all four restriction enzyme sites.

The repository of BioBrick parts at MIT's registry is fast growing. However, there are issues with concerning the functioning of some of these parts in the registry. These, together with other genetic parts that are of industrial and practical purposes such as those for solvent tolerance in bacteria should be properly characterised. This, when made available to the wider synthetic biology community can be further developed to improve the yield and titre of biofuels during bioprocessing.

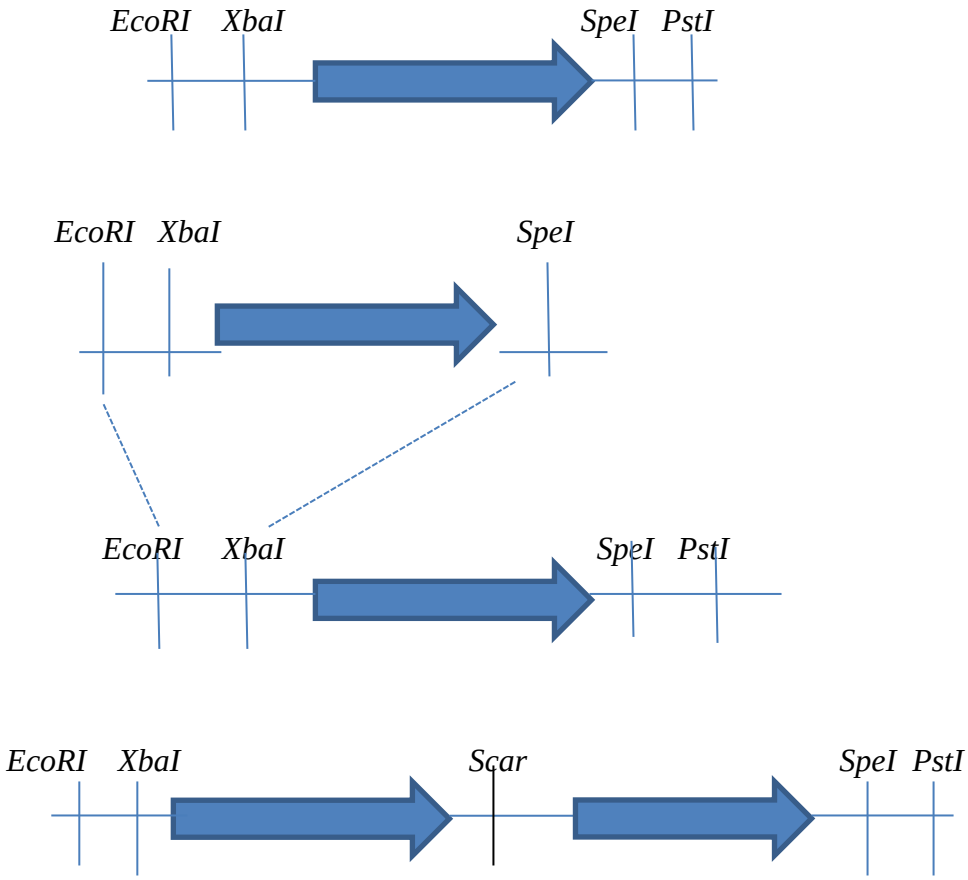


Figure 1.3: Assembly of BioBrick parts.

BioBrick parts are designed to have prefix and suffix sequences containing specific restriction sites. Using the right set of restriction enzymes, a BioBrick can be placed either upstream or downstream of an existing BioBrick to form a new part with both prefix and suffix sequences.

The need to further understand the effect of organic solvents on the microbial hosts cannot be overemphasised as discussed in this review. Synthetic biology also provides an opportunity to further investigate these mechanisms by assembling 'parts' comprising promoters, ribosome binding sites, fluorescent or luminescence reporter genes and terminators. Fluorescent- and luminescence-based reporters have been previously used to build bacteria biosensors which can detect metal ions and other toxic substances in the environment (Gu and Gil, 2001; Roda *et al*, 2011; Wang *et al*., 2013). In my view, these systems can also be used to visualise the effect of

solvents on cells in real time. For example, the *lux* operon (*luxCDABE*) from the marine bacterium, *Vibrio fischeri*, designated as BBa_K325909 in the Registry of Biological Parts encodes a luciferase enzyme which catalyses a series of reactions to produce light. The luciferase enzyme is a heterodimer made up of two subunits encoded by *luxA* and *luxB* respectively and catalyses this light forming reaction:

$FMNH_2 + O_2 + RCHO \rightarrow FMN + H_2O + RCOOH + photon$ (Meighen, 1994)

Three other proteins encoded by *luxCDE* catalyse a series of reactions which results in the conversion of tetradecanoic acid from the cell's fatty biosynthesis to form the aldehyde substrate used for the reaction (Meighen, 1994).

Using this principle, a bioluminescence assay can also be used to complement the traditional ways of assessing organic solvent tolerance which include plate assays, colony counts and reading absorbance of liquid cultures by using light production or inhibition of cultures exposed to a solvent as a measure of cell viability.

Finally, another practical way of using synthetic biology to solve the problems associated with organic solvent toxicity would be to build a network of genes implicated in solvent tolerance in suitable biological hosts. Due to the modularity and interchangeable nature of BioBrick parts, it will be possible to combine several of these genes in any order to build new parts which can be tested. Since solvent tolerance has been described as a complex phenotype involving several genes (Reyes *et al*. 2011) there is a likelihood of interaction and synergy between genes linked to different solvent tolerance mechanisms (French, 2009). Again, it would also be possible and interesting to use a combinatorial approach to increase solvent tolerance by combining solvent tolerance genes obtained from different organisms.

In summary, microbial production of biofuels is limited by the toxicity of these fuels to the microbial hosts making it a valid case to investigate the basis of toxicity in a bid to increase tolerance. Bacteria respond to toxic chemicals by turning several genes on or off in a concerted way. The role of most of these genes, individually or collectively, in solvent tolerance is still not fully understood. It will be interesting to employ synthetic biology tools and novel approaches to further investigate the effect of the solvents on the cell and the contribution of stress response genes to solvent tolerance. Knowing this will be an important step in designing rational strategies to increase tolerance to organic solvents.

1.14 Aims of this research

1. To identify genes (modules) suited for ethanol, *n*-butanol, furfural and acetone tolerance by employing different approaches (biomass measurements, colony counts and a bioluminescence assay) to screen a library of stress response genes.
2. To determine the effect of *n*-butanol on the cell envelope and morphology of *E. coli.*
3. To increase *n*-butanol tolerance by reducing membrane leakage through *cis-trans* isomerisation of the membrane lipids.

Chapter 2: Materials and Methods

2.1 Bacterial Strains and plasmids

The *E. coli* JM109 strain, (*endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB*$^{+}$ *Δ(lac-proAB*) e14- [*F' traD36 proAB*$^{+}$ *lacI*^{q} *lacZΔM15*]) a derivative of the K-12 strain and the wild type strain, MG1655 (*F*$^{-}$ *λ*$^{-}$ *ilvG*$^{-}$ *rfb-50 rph-1*) were used in this study. The plasmids, pSB1C3 and pSB4K5, were obtained from the Registry of Standard Biological Parts (http://partsregistry.org). Features of pSB1C3 (100 – 300 copies per cell) include a pUC19-derived pMB1 replication origin, chloramphenicol resistance gene and an efficient transcription terminator. pSB4K5 is a low copy vector (~ 5 copies per cell) with a pSC101 replication origin and a kanamycin resistance marker (see appendix for plasmid maps).

2.2 Media and reagents

The *E. coli* strains were aerobically grown in Luria-Bertani broth (LB), on solid LB agar or in M9 media supplemented with the appropriate antibiotics for selection.

2.2.1 Luria-Bertani (LB) Media

One litre of LB broth was made by dissolving 10 g Difco Tryptone, 10 g NaCl, and 5 g yeast extract in distilled water to a final volume of 1 litre (Bertani, 1951). This was then autoclaved prior to use. To make solid media, 1.5% w/v agar was added to the broth. The pH was adjusted to 7.2.

2.2.2 M9 media

To make 100 ml M9 media, the following components were mixed together: 25 ml 4 × M9 salts, 3.4 ml Thiamine hydrochloride (10 mg/ml), 10 µl 1M $CaCl_2$, 200 µl 1M

$MgSO_4$, 2 ml 20% glycerol and 2 ml 10% casamino acid. Sterile water was added to the 100 ml mark. All the components were sterilised by autoclaving apart from the Thiamine hydrochloride which was filter-sterilised.

4 × M9 salts: 28 g $NaHPO_4$, 12 g KH_2PO_4, 2 g NaCl, 4 g NH_4Cl dissolved in 1L distilled H_2O

2.2.3 Antibiotics and other reagents

The antibiotics used, chloramphenicol (40 µg/ml), kanamycin (50 µg/ml) and ampicillin (100 µg/ml) were obtained from Melford Laboratories (Suffolk). Isopropyl-β-D-thiogalactopyranoside (IPTG – 90 mg/ml) and X-gal (40 mg/ml) were also obtained from Melford Laboratories (Suffolk). Restriction endonucleases (*Eco*RI, *Spe*I, *Xba*I, *Pst*I,), T4 DNA ligase, T4 Polynucleotide kinase were purchased from New England Biolabs (NEB). KOD Hot Start DNA polymerase, its 10X reaction buffer and dNTPs (deoxynucleotide triphosphate) were from Novagen and used under conditions specified by the suppliers. The Phusion polymerase, obtained from New England Biolabs (NEB) was used to PCR amplify high GC templates. DNA digests and PCR products were purified using the Qiagen Purification kit according to the manufacturer's instructions. The organic solvents (ethanol, *n*-butanol and acetone) were from Fisher Chemicals (Loughborough). Furfural was purchased from Sigma-Aldrich (USA).

2.3 DNA Methods

2.3.1 PCR cloning and BioBrick construction

The primers used were designed to contain the BioBrick prefix bearing *Eco*RI and *Xba*I restriction sites in the forward primer and the BioBrick suffix bearing the *Spe*I

and *Pst*I restriction sites in the reverse primer. The native Ribosome Binding Sites (RBS) were included upstream of the coding region. The primers were ordered from Sigma-Adrich.

The genes used in this study were amplified by the Polymerase Chain Reaction (PCR) from *E. coli* MG1655 genomic DNA using the Peltier Thermal Cycler PTC-200 (MJ Research, Canada). The reaction was carried out according to the manufacturer's protocol. The *E. coli manXYZ* operon had two internal *Pst*I sites in the *manX* and *manY* genes which were removed by PCR mutagenesis in two steps using the *manX* and *manY* mutagenesis primers.

When the KOD polymerase was used, an initial denaturation was performed at 95^{o}C for 2 min followed by 35 cycles of denaturation at 95^{o}C for 20 sec, annealing for 10 sec depending on melting temperature of primers and primer extension at 70^{o}C. The extension time used was 10 sec/kb for fragments below 0.5 kb; 15 sec/kb for fragments of size 0.5-1 kb and 20 sec/kb for fragments of 1-3 kb.

To amplify high GC templates with the Phusion polymerase, an initial denaturation took place at 98^{o}C for 2 min followed by 35 cycles of denaturation at 98^{o}C for 30 sec, annealing for 20 sec depending on the melting temperatures of the primers and a primer extension at 72^{o}C. The extension time used was 1 kb per 30 sec.

Table 2.1 : List of genes and primers sequences

BioBrick part	Forward primer (5'-3')	Reverse primer (5'-3')
manXYZ	ATCGAATTCCTTCTAGAGATAATAAAGGAGGTAGCAAATGACCATTGCTATTGTT	ATCCTGCAGCTACTAGTATTATTACAGTCCCAGCAGGCC
manX (mutant)	GGAGCAGTTGCTTAAAACGG	GCAGCCCAACCATGTGTGCC
manY (mutant)	GGTTGCGCCTGACGCCGC	GCAGCACCGATGTTCATCC
glpC	ATCGAATTCCTTCTAGAGCAACGCGCAGGAGGCCAACAATGAATGACACCAGCTTC	ATCCTGCAGCTACTAGTATTATTAAGCCAGCGCCTGGGC
marA	ATCGAATTCCTTCTAGAGACAAAAAAGAGGTATGACGATGTCCAGACGCAATACT	ATCCTGCAGCTACTAGTATTATTAGCTGTTGTAATGATT
feoA	ATCGAATTCCTTCTAGAGTGAGAAACAAGTAGGCACCTATGCAATACACTCC	ATCCTGCAGCTACTAGTATTATTAACAGGAAACCGCTTCC
dps	ATCGAATTCCTTCTAGAGACTGGGACATAACATCAAGAGG	ATCCTGCAGCTACTAGTAGTTCTGCACCATCAGCGATGG
groESL	ATCGAATTCCTTCTAGAGCCGGCGTCACCCATAACAGATACGG	ATCCTGCAGCTACTAGTATTATTACATCATGCCGCCCATGCCACCC

The underlined bases are restriction sites used for cloning.

PCR products (5 µl) mixed with 5 µl sterile water and 2.5 µl loading dye were analysed by electrophoresis on an agarose gel containing 0.8% agarose in 1 × TAE (Tris-acetate-EDTA) buffer at 120 V and 50 mA for 30 min. The successfully amplified PCR products were then purified and subcloned into *Eco*RI/*Pst*I-digested pSB1C3 and pSB4K5 (Registry of Standard Biological Parts). However, *groESL* was *Eco*RI/*Spe*I digested and ligated into pSB1C3 since it had seven internal *Pst*I sites.

Loading dye: 0.6 ml 50% v/v glycerol, 0.3 ml 20 x TAE, and 0.1 ml 10% w/v SDS and a few grains of bromophenol blue

The oxidative stress response genes used in this study, *rcsA, sodA, katG* and *gorA*, were obtained from Nimisha Joshi (unpublished). These genes were originally amplified from *E. coli* MG1655. The *atf1* gene was acquired from the Registry of Standard Biological Parts (www.partsregistry.org) and the prefoldin construct, *phPFD* was received from the 2010 TU-Delft iGEM team (www.2010.igem.org)

The fatty acid *cis-trans* isomerase (*cti*) gene originally from *Pseudomonas putida* DOT-T1E was codon optimised for expression in *E. coli* (see appendix) and purchased from Life Technologies (Germany). The codon optimised sequence was designed to include the *E. coli* ribosome site, B0034 (www.partsregistry.org) upstream of the coding sequence.

2.3.2 Transformation and miniprep

E. coli chemically competent cells were prepared and transformed as described by Chung *et al*. (1989).

Plasmid DNA was prepared from 1.3 ml of overnight *E. coli* liquid cultures. These were centrifuged at 8000 × g for 3 minutes and supernatant was removed. The pellet

was resuspended in 100 µl of RNase A solution made from 5 µl/ml of 5 mg/ml RNase A stock solution. Solution 2 (200 µl of 0.2 M NaOH, 1% w/v SDS in H_2O) was added and mixed by inversion. Chilled Solution 3 (150 µl of 3 M potassium acetate, 2 M acetic acid) was added and mixed immediately by inversion. Cells were incubated on ice for 10 minutes and then centrifuged at 13 000 × g for 3 minutes. The supernatant was transferred to a new centrifuge tube avoiding the precipitate which was discarded. Absolute ethanol (920 µl) was added, mixed by inversion, and incubated on ice for 10 minutes. The mixture was centrifuged at 13 000 × g for 10 minutes to pellet the DNA and the supernatant was discarded. Ethanol (200 µl 70% v/v) was added to the pellet, centrifuged briefly and discarded. Plasmid DNA was dissolved in 40 µl elution buffer (10 mM Tris-HCl, pH 8) and mixed by pipetting. DNA was stored at -20ºC.

2.3.3 Addition of the *lac* promoter

The *lac* promoter (BioBrick BBa_J33207, which also includes the *lacZα'* gene) was *Eco*RI/*Xba*I digested, purified and ligated upstream of purified *Eco*RI/*Spe*I digested plasmid DNA containing the BioBrick. In both cases, digestion was carried out at 37 ^{o}C for 2 hr. The DNA fragments were ligated overnight at 16 ^{o}C. Competent *E. coli* JM109 cells were transformed by the heat shock procedure with plasmids containing the *lac* promoter upstream of the BioBrick part. Clones were selected on LB agar plates supplemented with IPTG (90 µg/ml), X-Gal (40 µg/ml) and appropriate antibiotics. Blue colonies were subcultured and a plasmid miniprep was performed as described earlier.

2.3.4 DNA sequencing

The BioBrick parts were sequence verified by the Gene Pool, University of Edinburgh using the Big Dye reaction with sequencing primers based on upstream (forward) and downstream vector sequence (reverse).

Forward primer sequence (pSBNX3 insf2): aaataggcgtatcacgaggc

Reverse primer sequence (pSBNX3insr2): cagtgagcgaggaagcctgc

2.4 Growth Assays

2.4.1 Viable cell counts (CFU)

Overnight cultures of the transformants and control were used to inoculate LB medium containing ethanol, *n*-butanol, acetone, or furfural and supplemented with 40 µg/ml of chloramphenicol and 90 µg/ml IPTG. The cultures were incubated at 37 ^{o}C for 24 hr. Cell densities of these cultures were measured at 600 nm (OD_{600}) using the Ultrospec III (Pharmacia LKB). Colony counts were also performed on the cultures to determine the actual number of cells that survived this toxic exposure. Samples were taken from cultures at the beginning of experiment and after 24 hours, serially diluted 10-fold in PBS (Phosphate buffered saline), plated on LB agar plates and incubated at 37^{o}C overnight. The number of colonies formed was counted. Results were obtained from three biological replicates each done with three technical replicates. Percentage survival for each strain was calculated as follows:

$$\% \text{ survival} = \frac{\text{CFU/ml (solvent added)}}{\text{CFU/ml (no solvent control)}} \times 100$$

2.4.2 Growth

To determine the effect of the organic solvents on the bacteria with increasing time, 200 µl of LB broth supplemented with the appropriate antibiotics, 90 µg/ml IPTG and overnight cultures diluted 1:100 into the fresh LB broth was dispensed into the wells of a 96-well plate (Greiner Bio-One, Germany). In order to avoid cross contamination of nearby wells and to prevent evaporation of the solvents, the plates were covered with a transparent adhesive film (Greiner Bio-One, Germany). The cultures were incubated at 37^{o}C (unless otherwise stated) in a Sunrise Blue microplate reader (Tecan Ltd, UK) for 24 hr with shaking at 150 rpm. Absorbance at 595 nm was measured every 15 min. The experiment was done three times to obtain three biological replicates.

2.4.3 Luminescence Assay

The *E. coli* host strain was sequentially co-transformed with *luxCDABE* in pSB4K5 and pSB1C3 carrying a potential solvent tolerance BioBrick. Overnight cultures were used to inoculate fresh M9 medium supplemented with 40 µg/ml chloramphenicol and 50 µg/ml kanamycin. IPTG (90 µg/ml) was added to induce the *lac* promoter upstream of the potential solvent tolerance BioBricks and 100 mM D-arabinose was used to induce the *pBAD* promoter driving the expression of the lux operon. Cultures were grown for 1 hr at 37 ^{o}C with shaking to enable the cells adapt to the medium and express the tolerance genes after which ethanol, *n*-butanol, furfural or acetone was added to the medium. After 5 hr of incubation, luminescence of the cultures was measured at room temperature using the Modulus Multimode reader (Turner Biosystems). Cell densities were also measured at OD_{600} using the Ultrospec III (Pharmacia LKB) spectrophotometer. Luminescence values are presented as relative

light units (RLU). The specific luminescence ratio of each strain was calculated as follows:

$$\text{Specific luminescence ratio} = \frac{\text{RLU/OD}_{600}\text{ (solvent added)}}{\text{RLU/OD}_{600}\text{ (no solvent control)}}$$

2.5 Enzyme assays

Samples used for the enzyme assays were prepared as follows. *E. coli* cells transformed with the *lacZα'* and *phoA* genes under the control of the *lac* promoter on pSB1C3 were grown overnight in 5 ml LB supplemented with 40 µg/ml chloramphenicol at 37 ^{o}C with shaking. Next, the overnight culture was diluted 1:100 into 20 ml fresh LB supplemented with 40 µg/ml chloramphenicol and 90 µg/ml IPTG and incubated at 37 ^{o}C for 3 hr till the culture attained an OD_{600} of 0.5-0.6. The liquid culture was then split into two groups. Each group was made up of four 2.5 ml cultures to which 1% *n*-butanol, 8 mM EDTA, 8 mM SDS or water (no solvent control) was added. Cultures containing these chemicals were incubated for 1 hr at 37 ^{o}C after which they were centrifuged to separate the cells from the liquid media. As stated before, the original 25 ml culture was split into two groups. In one group, the supernatant was used for the enzyme assay and in the other group, a suspension of whole cells were used. When using whole cells, the supernatant was removed and the cells were washed twice and resuspended in PBS.

2.5.1 Alkaline phosphatase assay

Alkaline phosphatase activity was determined in a reaction mixture of 1.25 ml containing: 0.05 M Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 50 mM NaCl, 125 µl of sample and 20 mM *p*-Nitrophenyl Phosphate (p-NPP) substrate. In the experiment to determine the effect of 8 mM EDTA on the outer membrane, a higher concentration

(40 mM) of $MgCl_2$ was used to prevent EDTA from inhibiting the alkaline phosphatase. The reaction mixture was incubated at 37^{o}C until a yellow product was formed. The time taken for the mixture to turn yellow was noted. The end-point measurement was used because the supernatants of EDTA or butanol-treated cultures had varying amounts of alkaline phosphatase so detectable product formation occurred at different times. The reaction was stopped with 500 µl of 1 M Potassium phosphate. The absorbance of the reaction mixture was measured at 410 nm (A_{410}) with Ultrospec III (Pharmacia LKB) spectrophotometer.

$$\text{Enzyme activity was determined as: } 1000 \times \frac{A_{410}}{OD_{600} \times \text{volume of sample (ml)} \times \text{incubation time (min)}}$$

2.5.2 Miller Assay

The β-galactosidase assay (Miller assay) was performed as described by Miller (1972) with some slight modifications. The substrate solution was made up of 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and 19.5 mM dithiothreitol (DTT). For the reaction, 100 µl of sample (supernatant or whole cells) was added to 600 µl of the substrate solution pre-warmed at 30^{o}C. The reaction mixture was incubated at 30^{o}C until it turned yellow signifying product formation. The reaction was then stopped with 700 µl of 1M Sodium carbonate (Na_2CO_3). Absorbance readings were taken at 420 nm with the Ultrospec III (Pharmacia LKB) spectrophotometer and the enzyme activity was calculated and expressed in Miller units.

$$\text{Miller unit} = \frac{A_{420}}{OD_{600} \times \text{volume of sample (ml)} \times \text{incubation time (min)}}$$

2.6 Characterisation of the *lptA* bioreporter

2.6.1 Construction of the *lptA* bioreporter

The *lptA* promoter was amplified by PCR from the *E. coli* MG1655 genome using the Peltier Thermal Cycer PTC-200 (MJ Research, Canada) and was obtained in the BioBrick format. The PCR was carried out with the high fidelity Phusion DNA polymerase from New England Biolabs Inc. (NEB). The following pair of primers was used:

Forward: ATCGAATTCCTTCTAGAGATAACGCGCAGATCAATCTGGTGACGC

Reverse: ATCCTGCAGCTACTAGTAGGATGTTCTAACCTTTTCAATCAGCTCGGCG

The forward primer was designed to include the BioBrick prefix while the reverse primer included the BioBrick suffix sequence. The primers were synthesised by Sigma-Aldrich. The *lptA* promoter was placed upstream of a ribosome binding site (RBS) and a red fluorescent protein (RFP). Both RBS and RFP were obtained from the Registry of Standard Biological Parts (www.partsregistry.org) as BBa_B0034 and BBa_E1010 respectively. The *lptA* promoter + RFP construct was *Eco*RI/*Pst*I digested and ligated into plasmid pSB1C3. The bioreporter in pSB1C3 was sequenced verified using sequencing primers as shown earlier.

2.6.2 Bioreporter induction assay

Overnight cultures were used to inoculate fresh M9 media supplemented with 40 µg/ml chloramphenicol. The cells were grown for 4 hr at 37^{o}C with shaking after which cell growth (OD_{600}) and fluorescence were measured with the Modulus Multimode reader (Turner Biosystems) and the Ultrospec III (Pharmacia LKB) spectrophotometer respectively. Different concentrations of *n*-butanol or EDTA were added to cultures and absorbance at 600 nm together with fluorescence outputs

representing induction of the *lptA* promoter were then measured every 30 min for 2 hr.

2.7 Lipopolysaccharide (LPS) assay

Overnight cultures of *E. coli* MG1655 were used to inoculate fresh LB in a 1:100 ratio. The cells were incubated at 37^{o}C for 3 hr with shaking until they reached an OD_{600} of ~0.7. The cells were harvested by centrifugation, washed three times and then resuspended in 5 ml PBS to remove any residual growth media. *n*-butanol or EDTA was added to 1 ml of the cell suspension and incubated with shaking for 1 hr. The cell suspension was centrifuged at high speed (13 000 × g) for 3 min to obtain the supernatant containing released LPS for the assay.

The purpald assay was used to quantify LPS released from the outer membrane as described by Lee and Tsai (1999) with some slight modifications. All the reagents used for the assay were freshly prepared before use. A volume of 250 µl of the supernatant obtained earlier was added to 250 µl of 32 mM sodium periodate ($NaIO_4$) and incubated for 25 min. Then, 250 µl of 136 mM purpald (4-amino-3-hydrazino-1,2,4-triazol-5-thiol) was added and incubated for 20 min followed by the addition of 250 µl of 64 mM NaIO to the reaction mixture. After incubating for another 20 min, the absorbance was measured at 550 nm with the Ultrospec III (Pharmacia LKB) spectrophotometer.

2.8 Protein Assays

2.8.1 Isolation of periplasmic and cytosolic proteins

Overnight cultures of *E. coli* MG1655 were used to inoculate 250 ml LB supplemented with chloramphenicol (40 µg/ml) in a 1:100 dilution. The cultures were incubated at 30^{o}C with shaking (150 rpm) and induced with IPTG (90 µg/ml)

when they attained an OD_{600} ~ 0.600. These were further incubated for 4 hr after which the cells were harvested by centrifugation 10 000 × g for 20 min using the Sorvall RC-5B refrigerated super speed centrifuge with GSA rotor (Du Pont Instruments). Periplasmic proteins were isolated as described by Hong *et al*., (2007). The cell pellet was resuspended in 2 ml 20% sucrose - 0.03 M Tris-HCl (pH 8.0). Subsequently, 500 µl of 5 mM disodium EDTA (pH 8.0) was added and the resulting suspension was mixed for 10 min at room temperature. The cells were pelleted at 8000 rpm for 3 min and the supernatant was removed. The pellet was then resuspended in 1.25 ml ice cold distilled water and mixed for 10 min on a rotary shaker (180 rpm) at 4^{o}C. Afterwards, the cell suspension was centrifuged at 13 000 rpm for 1 min and the supernatant containing the periplasmic fraction was used for subsequent protein assays.

The pellet that remained was sonicated, centrifuged at 13 000 rpm for 3 mins and the supernatant containing the cytosolic fraction was removed and stored for subsequent assays. The cell debris was resuspended in 2 ml PBS and was also stored for future use.

2.8.2 Bradford assay

Protein concentration of the cell debris, periplasmic and cytosolic fractions was determined with the Bradford Assay (Bradford, 1976). To obtain a standard curve, 0, 0.2, 0.4, 0.6 and 0.8 mg/ml of Bovine Serum Albumin (BSA) Fraction V was added to 1 ml Bradford Reagent, mixed and the absorbance at 595 nm was obtained for each standard. The absorbance readings at 595 nm were then plotted against the concentration of the standards.

To determine the concentration of protein in the cell fractions, appropriate amounts of the periplasmic fraction, cytosolic fraction and cell debris was added to 1 ml of the Bradford Assay Reagent (Thermo Scientific, USA) respectively and mixed. Absorbance readings were measured at 595 nm and by extrapolating on the standard curve, the protein concentration of the samples was obtained. This was done twice and the average concentration of proteins was obtained.

2.8.3 SDS-PAGE

Proteins in the cell debris, periplasmic and cytosolic fractions were separated and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12 % acrylamide gel. Gels were cast in glass plates for use in a Biorad Mini Protean Tetra Cell gel tank. A total volume of 20 ml resolving gel was made by mixing 6.6 ml H_20, 8 ml of 30 % acrylamide mix (BioRad), 5 ml 1.5 M Tris (pH 8.8), 200 µl of 10 % SDS, 200 µl of 10 % ammonium persulphate and was polymerised with 8 µl TEMED (Tetramethylethylenediamine). The resolving gel was carefully pipetted into the space between the glass plates, leaving enough space for the stacking gel. The resolving gel was layered with 250 µl of isopropanol and allowed to set.

After setting, the isopropanol was removed and the stacking gel was poured above the resolving gel. Approximately, 5 ml of 5 % stacking gel was made by mixing together 3.4 ml H_2O, 0.83 ml of 30 % acrylamide mix, 0.63 ml of 1M Tris (pH 6.8), 50 µl of 10 % SDS, 50 µl of 10 % ammonium persulphate and 5 µl of TEMED. A clean Teflon comb was inserted into the stacking gel to make wells for loading the protein samples. The comb was removed when the gel had polymerised.

The protein samples were mixed with 1 × SDS gel loading buffer (50 mM Tris-HCl, 100 mM dithiothreiol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and boiled for 3 min before loading into the gel. Gels that were to be used for haem staining were boiled under non-reducing conditions ie dithiothreitol was not included in the SDS gel loading buffer in order not to dissociate the haem group (cytochrome c) from the haem-containing protein.

The gel was run at 80 V for 15 min and then at 200 V for 25 min in an electrophoresis tank containing 1 × running buffer (25 mM Tris, 250 mM glycine and 0.1% SDS). A prestained broad range protein marker (New England Biolabs) was run alongside the protein samples. The gel was then washed with distilled water to remove any remaining SDS and was stained in a Coomassie blue solution (0.25 g Coomassie brilliant blue R-250 dissolved in 250 ml methanol, 100 ml glacial acetic acid and 450 ml ddH_20) overnight after which the excess stain was washed off by rinsing the gel in a destaining solution (250 ml methanol, 100 ml glacial acetic acid and 450 ml ddH_20) for 3 hr.

2.8.4 Haem staining

This technique was used to identify the haem-containing protein, Fatty acid *cis-trans* isomerase (CTI) by staining with TMBZ (3,3',5,5'-tetramethylbenzidine) as described by Stugard *et al*., (1989) with some modifications. The proteins were run on an SDS-PAGE gel under non-reducing conditions. The gel was washed with distilled water to remove excess amounts of SDS and was placed in a 50 ml solution containing 35 ml 0.25M sodium acetate (pH 5.2) and 6.3 mM TMBZ dissolved in 15 ml methanol. The gel was incubated in this solution with mixing for 2 hr in the dark. Afterwards, 150 µl of 30% H_2O_2 was added to the solution and further incubated for 30 min in the dark. The solution was removed and the gel was washed three times

with a mixture of isopropanol and 0.25 M sodium acetate (pH 5.2) in a ratio of 3:7 to fix the stain and remove excess amounts of the TMBZ. The gel was rinsed and the bands were visualised.

2.9 Microscopy

E. coli MG1655 cells transformed with an enhanced green fluorescent protein (EGFP) on a pWR20 plasmid (kindly gifted by Teuta Pilizota, University of Edinburgh) were grown in LB supplemented with 50 µg/ml kanamycin at 37^{o}C till the cultures reached an OD_{600} of 0.5. The microscope slide was prepared by creating a centimetre-wide tunnel with a scotch permanent double-sided tape. A cover slip was placed over the tunnel and the tunnel was washed with polylysine. The polylysine-coated tunnel was washed with the bacterial culture to fix the cells. Excess cells were washed off with LB and the cells were incubated for 10 min at room temperature.

Different slides were prepared for different concentrations of *n*-butanol. The tunnel of each slide was flushed with a different concentration of *n*-butanol to expose the cells to the solvent and visualised with a Nikon fluorescence microscope with images taken every minute for 5 min.

2.10 Statistical Analysis

All the experiments were carried out in triplicate. Data were statistically analysed with an unpaired *t*-test and a *p*-value at 95% confidence intervals was calculated to demonstrate statistically significant difference in the means of the samples.

Chapter 3: Investigating solvent tolerance in *E. coli* with a multiple-assay approach

3.1 Introduction

Microbial production of fuels and other chemicals at high titres and yields has become a challenge due to the toxicity posed by these chemicals to the microbial hosts. During microbial conversion of biomass to biofuels, such as ethanol and butanol, growth inhibition is not only caused by the final products formed but also by other inhibitory substances such as furfural and 5-hydroxymethyl furfural produced during biomass pre-treatment. Organic solvent toxicity therefore limits the product titres from such biological processes resulting in high recovery costs to concentrate the products obtained. The most common method used to mitigate this issue of toxicity is the use of gas stripping to remove the products as they form and this has been shown to improve product yield (Xue *et al*., 2012). However, this might further add to the cost involved in this bioprocess. Distillation, which can be used to obtain high concentrations of the product, is also very expensive (Alzate and Toro, 2006) especially when the titre of the final product is low. To overcome these bottlenecks, it has become expedient to engineer robust microbial hosts suitable for these processes.

Considerable effort has gone into developing solvent tolerant microbes by using evolutionary approaches, random mutagenesis, adaptation and selection (Basak *et al.*, 2012; Horinouchi *et al*., 2010; Minty *et al*., 2011). Using this approach, *E. coli* mutants that survived up to 150 g/l ethanol (Chong *et al*., 2013) and 2% w/v isobutanol (Minty *et al*., 2011) were isolated. However, these approaches are time-consuming and depending on the sampling time, some important mutations may be

missed (Reyes *et al*., 2012). Therefore, a better alternative will be the use of a rational approach to engineer robust microbial hosts.

Rational engineering of the solvent tolerance phenotype requires a detailed understanding of the mechanisms of toxicity and tolerance. Advances in the development of genetic tools have made it possible to identify which genes respond to organic solvent stress leading to the generation of a vast amount of information on possible tolerance genes (Goodarzi *et al*, 2010). Recent reports indicate that most genes that respond to butanol stress have membrane-related functions and butanol stress elicits other stress responses such as heat shock, envelope stress and oxidative stress (Reyes *et al*., 2011; Rutherford *et al*., 2010). A combination of computation and experimentation was used to understand the genetic basis of ethanol tolerance (Goodarzi *et al*., 2010). Using this approach, it was found that genes that respond to ethanol stress are mainly involved in heat shock response, assimilation of ethanol and biosynthesis of glycine-betaine which acts as an osmoprotectant. Fermentation inhibitors such as furfural, which are produced during biomass pre-treatment, have been shown to inhibit key glycolytic enzymes in micro-organisms as well as deplete the cell's NADPH pool (Banerjee *et al*., 1981).

The solvent tolerance phenotype is very complex involving several mechanisms. Many genes have been shown to respond to stresses imposed on the cell by different organic solvents. However, the fact that a gene responds to a particular stress does not necessarily mean it increases tolerance of the host cell. It is important to tease out these parts to know the exact role each gene plays in solvent tolerance. The first step in this direction is to develop quick and reliable screening methods that can accurately determine cell survival after exposure to different inhibitory substances. In

this chapter, the use of a bioluminescence assay as an alternative assay to complement the traditional methods of measuring solvent tolerance will be demonstrated. As proof-of-concept, certain genes which have been previously reported to increase tolerance to different stresses were screened against four inhibitory substances – ethanol, *n*-butanol, furfural and acetone. Using synthetic biology tools, it was possible to generate standardised modules (BioBricks) of these potential solvent tolerance genes which can be combined in any arbitrary order to test for possible synergies. Synergistic interactions between combinations of these stress response genes will also be discussed in this chapter.

3.2 Results

3.2.1 Generating a BioBrick library of stress response genes

The first question asked was whether over-expressing stress response genes would protect cells from the toxicity of organic solvents such as ethanol, *n*-butanol, fermentation inhibitors and acetone. These chemicals are of industrial relevance and are produced during biomass conversion to biofuels. If tolerance was achieved by overexpressing these genes, which sets of genes would increase tolerance to each of these solvents? To address this, a library of possible solvent tolerance genes (Table 3.1) was generated in the BioBrick format (Knight, 2003), each gene was cloned into pSB1C3 and transformed it into an *E. coli* host strain. The rationale behind the choice of these genes is that nearly all of them have been shown from different transcriptional studies to be highly upregulated during microbial stress and many of them have not been previously tested against ethanol, *n*-butanol, furfural and acetone. Also, since they increase tolerance *via* different mechanisms, it will be possible to deduce which tolerance mechanisms are more relevant when cells are

exposed to these chemicals. Even though transcriptional studies are useful for identifying changes in gene expression following solvent stress, the fact that a gene is upregulated during stress does not necessarily mean it alleviates the stress. This can be known only by testing and experimentation.

3.2.2 Preliminary experiments to test for ethanol and *n*-butanol tolerance

Preliminary experiments were carried out to determine the effect of *n*-butanol and ethanol on *E. coli* growth using the JM109 strain since it is sensitive to stresses including oxidative stress and this strain of *E. coli* also ensures plasmid stability. Similar studies have used this strain for the same purpose (Ni *et al*., 2013; Pan *et al*., 2009). These cells were transformed with the potential solvent tolerance genes designed in the BioBrick format and were screened in increasing concentrations of ethanol (0%, 2%, 4%, 6%, 8% and 10% v/v) and *n*-butanol (0%, 0.1%, 0.5%, 1% and 2% v/v) to obtain initial data on the cell's ability to resist the toxic effect of these solvents. Control cells carried the pSB1C3 plasmid containing the *lac* promoter. The cultures were grown at 37 ^{o}C and absorbance readings at 600 nm (OD_{600}) were used to monitor growth inhibition after 24 hours of incubation.

The cell densities of the transformed cells and the control strain declined with increasing concentration of ethanol and *n*-butanol signifying growth inhibition with increase in solvent concentration (Figure 3.1). When exposed to ethanol, the OD_{600} reading of the liquid cultures significantly dropped for most of the strains at a concentration between 4% and 6% v/v (Figure 3.1a). A similar decline in growth was observed with increasing concentrations of *n*-butanol (Figure 3.1b). Cell growth declined sharply at a concentration between 0.5% and 1% v/v *n*-butanol. There was no further growth beyond these final concentrations.

Table 3.1: BioBrick Library of potential solvent tolerance genes

	Gene	Gene Product	Source (organism)	Reference
Coding membrane-associated proteins	*manXYZ*	Mannose-specific transporter	*E. coli*	Okochi *et al*., (2007)
	glpC	Anaerobic glycerol-3-phosphate dehydrogenase subunit	*E. coli*	Shimizu *et al*., (2008)
	feoA	Ferrous iron transport protein	*E. coli*	Reyes *et al*., (2011)
Stress response genes	*katG*	Catalase	*E. coli*	Michan *et al*., (1999)
	sodA	Superoxide dismutase	*E. coli*	Michan *et al*., (1999)
	gorA	Glutathione oxidoreductase	*E. coli*	Michan *et al*., (1999)
	dps	DNA-binding protein	*E. coli*	Michan *et al*., (1999)
Chaperone protein	*phPFD*	protein chaperone, Prefoldin which corrects protein misfolding /unfolding	*Pyrococcus horikoshii OT3*	Okochi *et al*., (2008)
Others	*atf1*	Alcohol acetyltransferase 1 which converts primary alcohols to acetyl esters	*Saccharo-myces cerevisiae*	http://partsregistry.org/Part:BBa_J45199
	rcsA	Exopolysaccharide biosynthesis regulator	*E. coli*	Ebel and Trempy, (1999)
	marA	DNA-binding transcriptional activator involved in antibiotic resistance	*E. coli*	Asako *et al*., (1997)

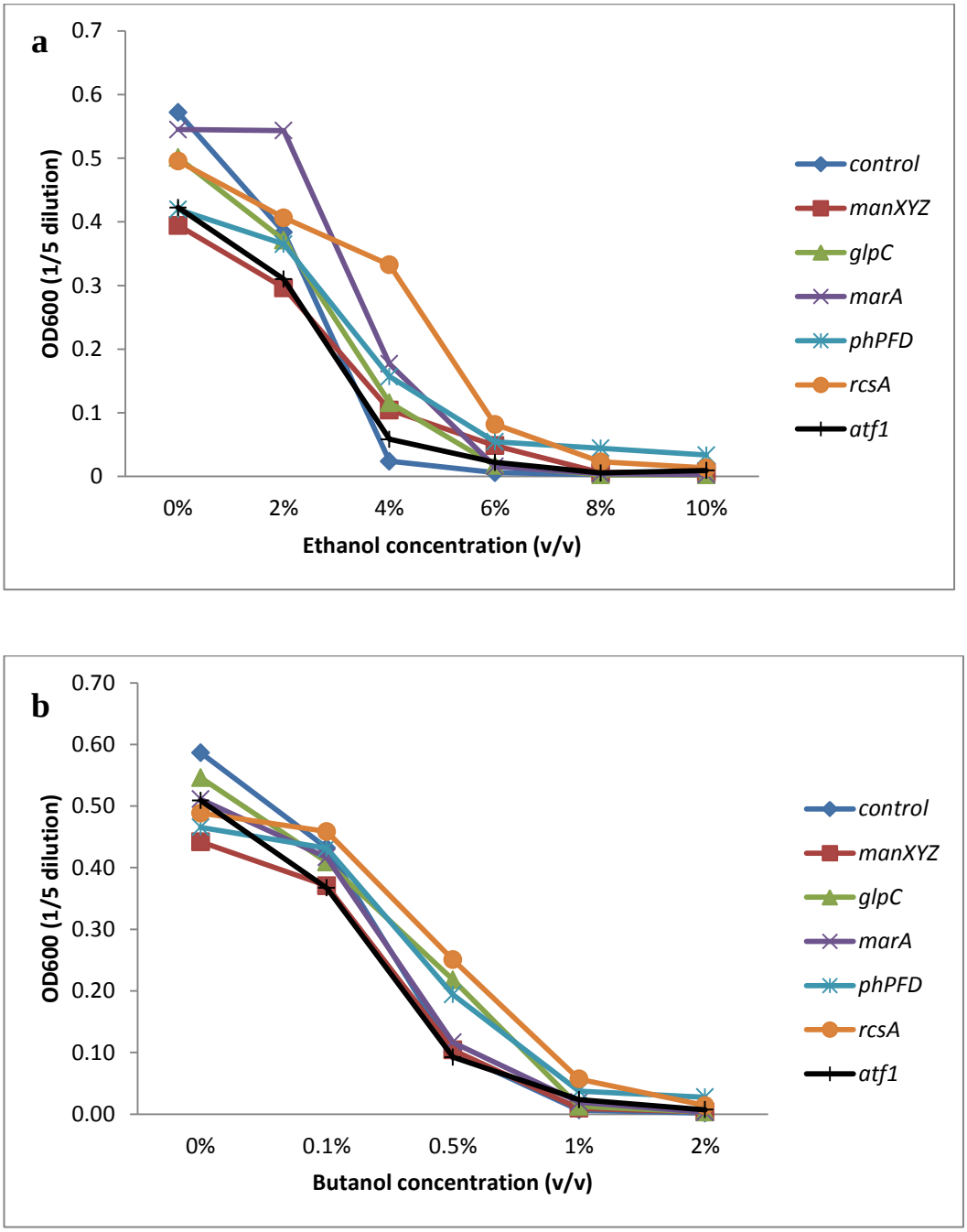


Figure 3.1: Preliminary growth inhibition experiments.

The effect of increasing concentrations of (a) ethanol and (b) *n*-butanol on *E. coli* JM109 overexpressing different stress tolerance BioBricks after a 24 hr incubation. The control strain carried an empty plasmid. Graphs show results from two biological replicates.

It was also important to determine the exact concentration (maximum tolerable concentration) of these solvents that will completely inhibit cell growth. To do this, the range of ethanol concentrations used for the screening was narrowed to 5.5%, 6%, 6.5% and 7% (v/v) while that of *n*-butanol was narrowed to 0.7%, 0.8%, 0.9% and 1% (v/v). The inhibitory effect of ethanol was pronounced at 6% (v/v) beyond which cells completely stopped growing (Figure 3.2a). In the *n*-butanol experiment, 0.9% (v/v) *n*-butanol was the maximum concentration that could be tolerated by the cells (Figure 3.2b). Since growth was completely inhibited by 0.9% *n*-butanol and 6% ethanol, a milder concentration of these solvents was used throughout the following experiments in order to obtain a more observable difference in relative tolerance of the different strains.

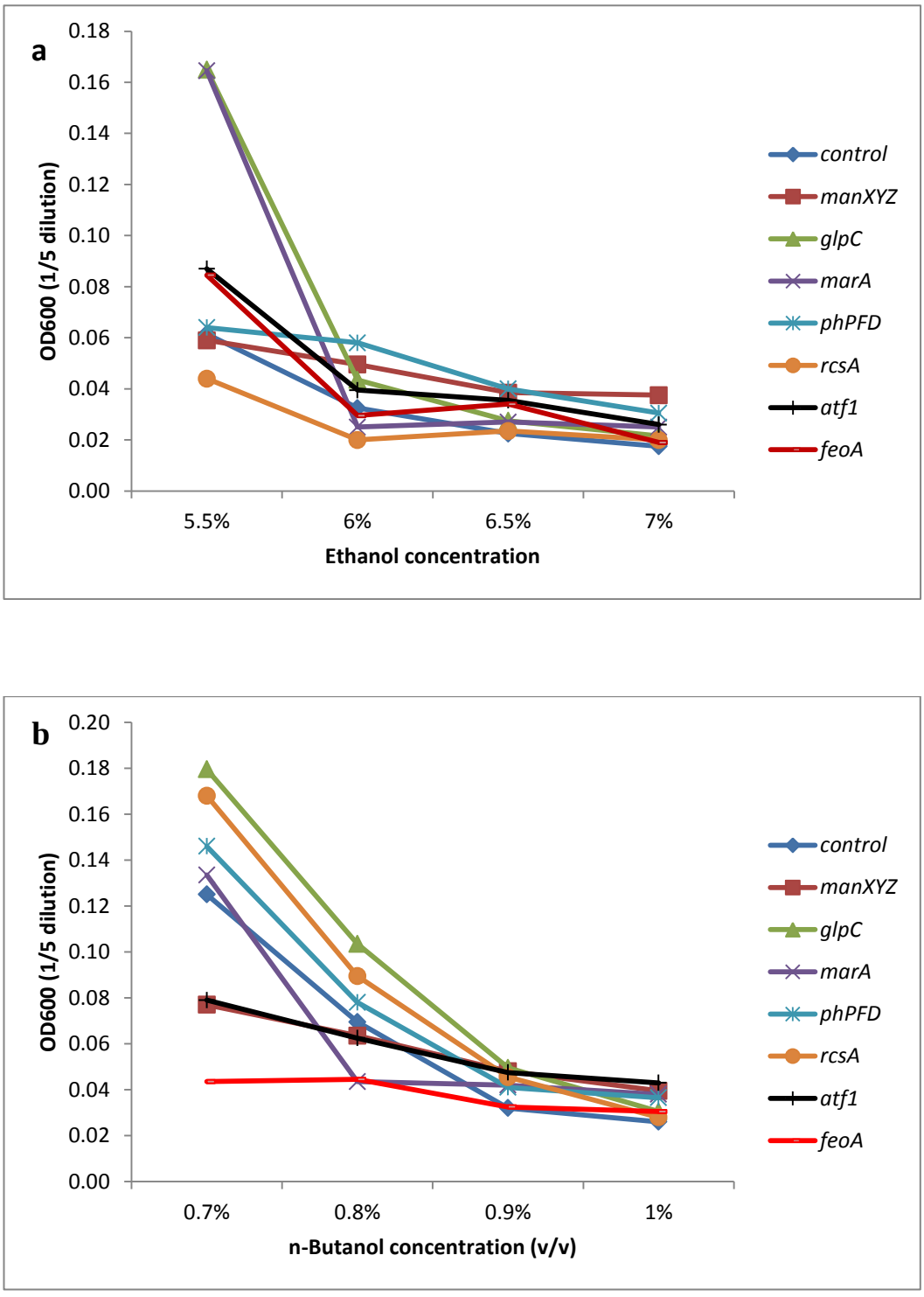


Figure 3.2: Determination of the maximum tolerable concentration for *E. coli* JM109 and the recombinant strains to (a) ethanol and (b) *n*-butanol. The control strain carried an empty plasmid. Graphs show results from two biological replicates.

3.2.3 Determination of solvent tolerance with different approaches

3.2.3.1 Cell growth assay

The BioBrick library was screened for ethanol and *n*-butanol tolerance by growing the cells in a mild concentration of these alcohols for 24 hr. The final optical density (OD_{600}) of the cultures was used to determine growth of the individual strains at this concentration of solvent. Using this assay to determine tolerance, seven genes (*dps, phPFD, rcsA, feoA, manXYZ, marA* and *katG*) in the library were found to increase tolerance to 4% ethanol ($p < 0.05$). The remaining four, *glpC, sodA, atf1* and *gorA*, did not increase tolerance to ethanol (Figure 3.3). The cell densities of the different strains were identical to that of the control in the absence of ethanol and *n*-butanol.

Using the same method, genes conferring *n*-butanol tolerance were also identified after a 24 hr incubation in 0.5% *n*-butanol at 37^{o}C. Cells overexpressing *phPFD, marA, atf1, dps* and *katG* showed enhanced growth in *n*-butanol compared to the control strain ($p < 0.05$). However, those overexpressing *sodA* and *gorA* were more sensitive to *n*-butanol compared to the control strain.

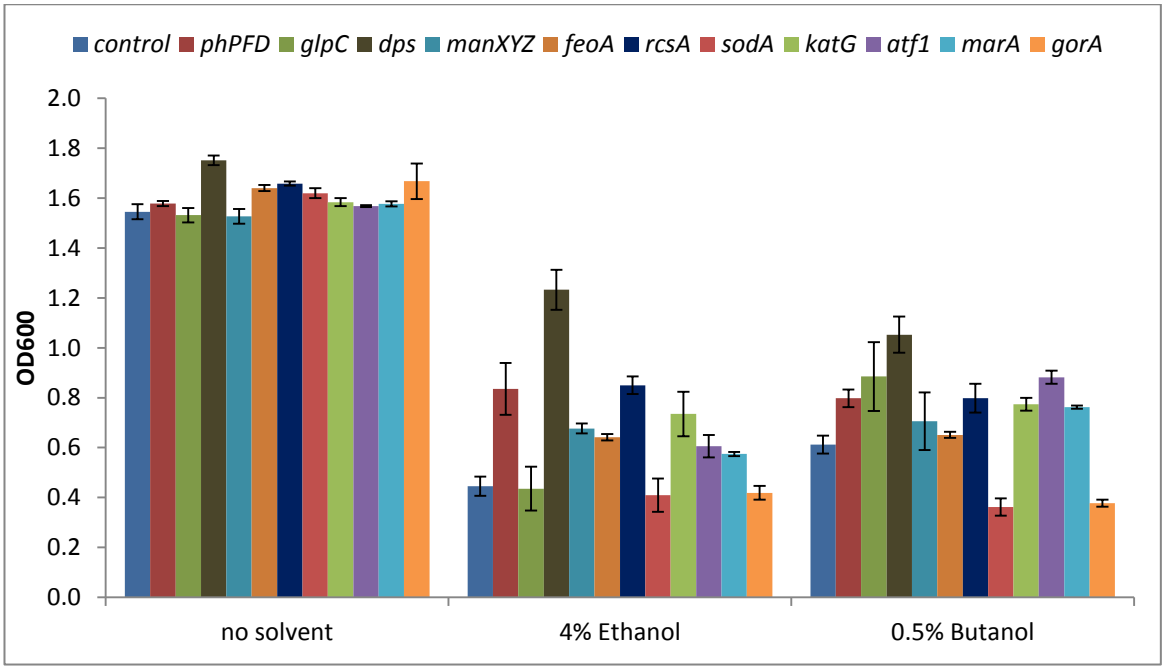


Figure 3.3: Effect of different stress tolerance genes on *E. coli*.

A growth assay performed after 24 hr of incubation by measuring absorbance at 600 nm was used to determine tolerance to ethanol and to *n*-butanol. Error bars show standard error between three biological replicates.

3.2.3.2 Colony forming unit (CFU/ml)

Using another method, the effect of both alcohols on the different strains was quantitatively determined. By performing colony counts, it was possible to obtain an approximate number of cells that survived the toxic exposure after 24 hr of incubation. The percentage survival of each of the strains was calculated as described in the Materials and Methods Section (Chapter 2).

Cell viability was severely reduced at these concentrations of ethanol and *n*-butanol. However, in ethanol, cells overexpressing the protein chaperone, *phPFD* had the highest percentage survival which was a significant improvement in tolerance ($p<0.05$) compared to the control strain. To a lesser extent as shown in Figure 3.4a, those overexpressing *glpC, manXYZ, feoA, sodA* and *marA* survived better than the control strain ($p<0.05$). Interestingly, *rcsA, dps* and *katG* were unable to increase tolerance ($p > 0.05$) contrary to the earlier OD_{600} data presented in Figure 3.3. Also, other genes such as *glpC* and *sodA* which did not show tolerance to ethanol in the growth assay (Figure 3.3) showed a significant increase in tolerance using the colony count.

Assaying for *n*-butanol tolerance, Figure 3.4b shows that *phPFD, glpC, atf1* and *marA* had a significantly higher percentage survival compared to the control strain ($p < 0.05$). Cells carrying the *phPFD* gene had the highest percentage survival of 25% ($p = 0.001$) at this concentration of *n*-butanol suggesting an element of butanol-induced protein damage during butanol stress. Cells overexpressing *feoA* had a relatively higher percentage survival compared to the control. The difference, however, was not significant ($p = 0.051$) probably due to large variations in the triplicate experiments resulting in the huge standard error. Consistent with the OD_{600} readings, the *gorA* strain showed a reduced percentage survival compared to the

control strain. Again, in contrast to the OD_{600} readings, overexpressing *dps,* and *katG* did not increase tolerance to *n*-butanol.

3.2.3.3 Assessing solvent tolerance with a bioluminescence assay

Standard methods for assaying cell growth involve overlaying a solvent onto a medium agar plate, taking absorbance readings at 600 nm, colony counts and plate assays. These methods have several limitations characterised by inconsistent results and lack of sensitivity (Kataoka *et al*., 2011) and can be laborious. As an alternative to the previous assays used to determine solvent tolerance, a bioluminescence-based method was employed to further screen the library of stress tolerance genes. This method involved screening cells co-expressing the bacterial luciferase *luxCDABE* and a potential solvent tolerance gene on two compatible plasmids (see Materials and Methods) in the different solvents for tolerance. A measurement of the intensity of bacterial light produced was used to assess tolerance.

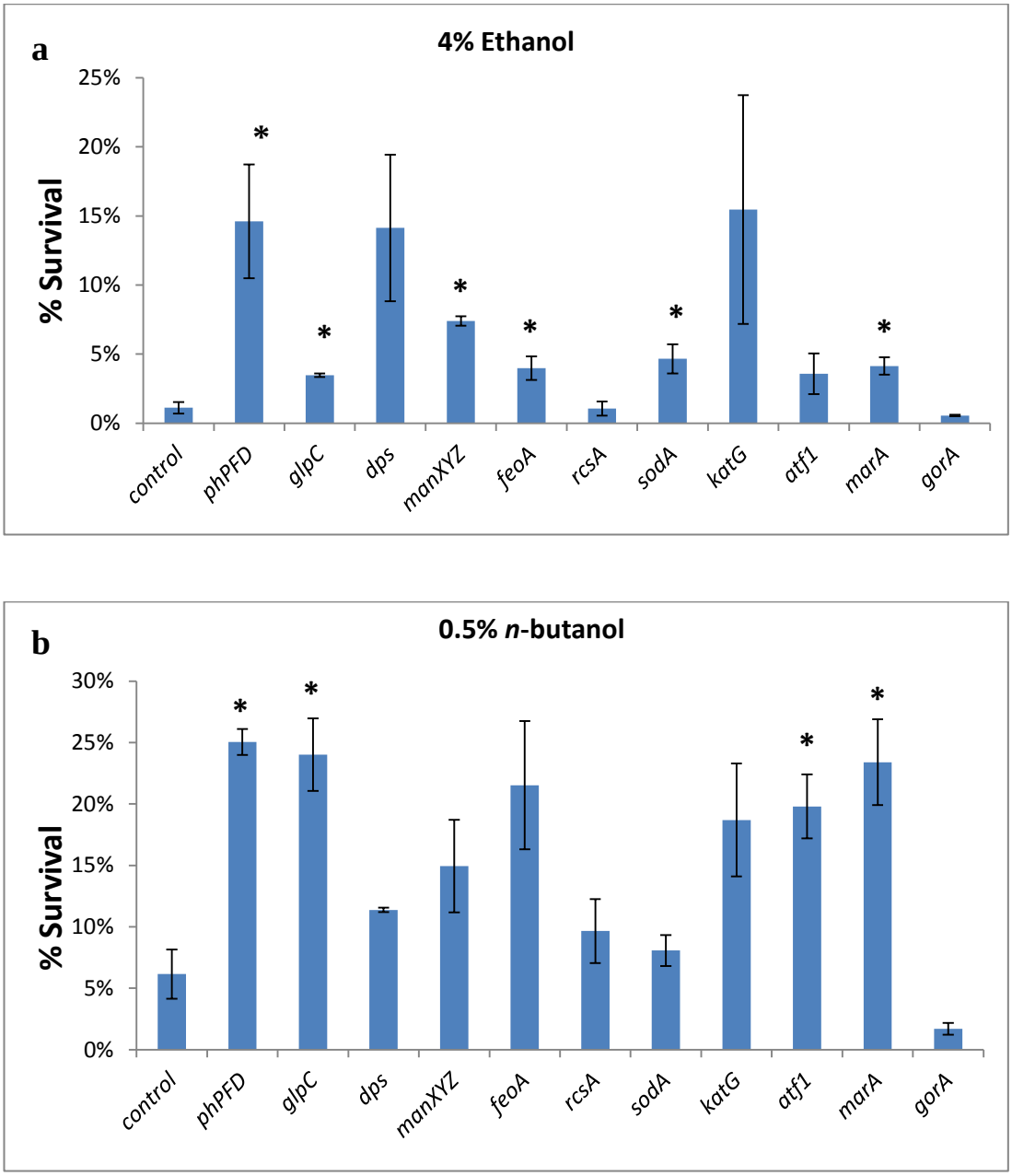


Figure 3.4: Determination of solvent tolerance with a viable cell count (CFU/ml).

Tolerance of the different strains of *E. coli* to (a) ethanol and (b) *n*-butanol was determined by performing colony counts after 24 hr exposure. The number of colonies counted per ml was used to determine the percentage survival. The error bars represent standard error of three biological replicates. Asterisks indicate statistical significance (p <0.05).

A time-course experiment was conducted to determine the half-life of the luminescence emitted by cells expressing the *lux* genes. Luminescence and OD_{600} readings were taken every hour for eight hours and after 24 hr of incubation. Cells carrying pSB4K5 without the *lux* genes were used as control (Figure 3.5a). Luminescence increased with time until it peaked after 5 hr of incubation and then began to drop with increasing time until 24 hr where there was no longer any luminescence even though the OD_{600} of the cultures increased progressively till they reached the stationary phase at 5 hr. The control cells did not produce any luminescence. Therefore, further experiments were carried out by measuring luminescence at 5 hr. It was also observed that luminescence was correlated to cell density. (Figure 3.5b) making this assay useful for measuring cell viability and survival.

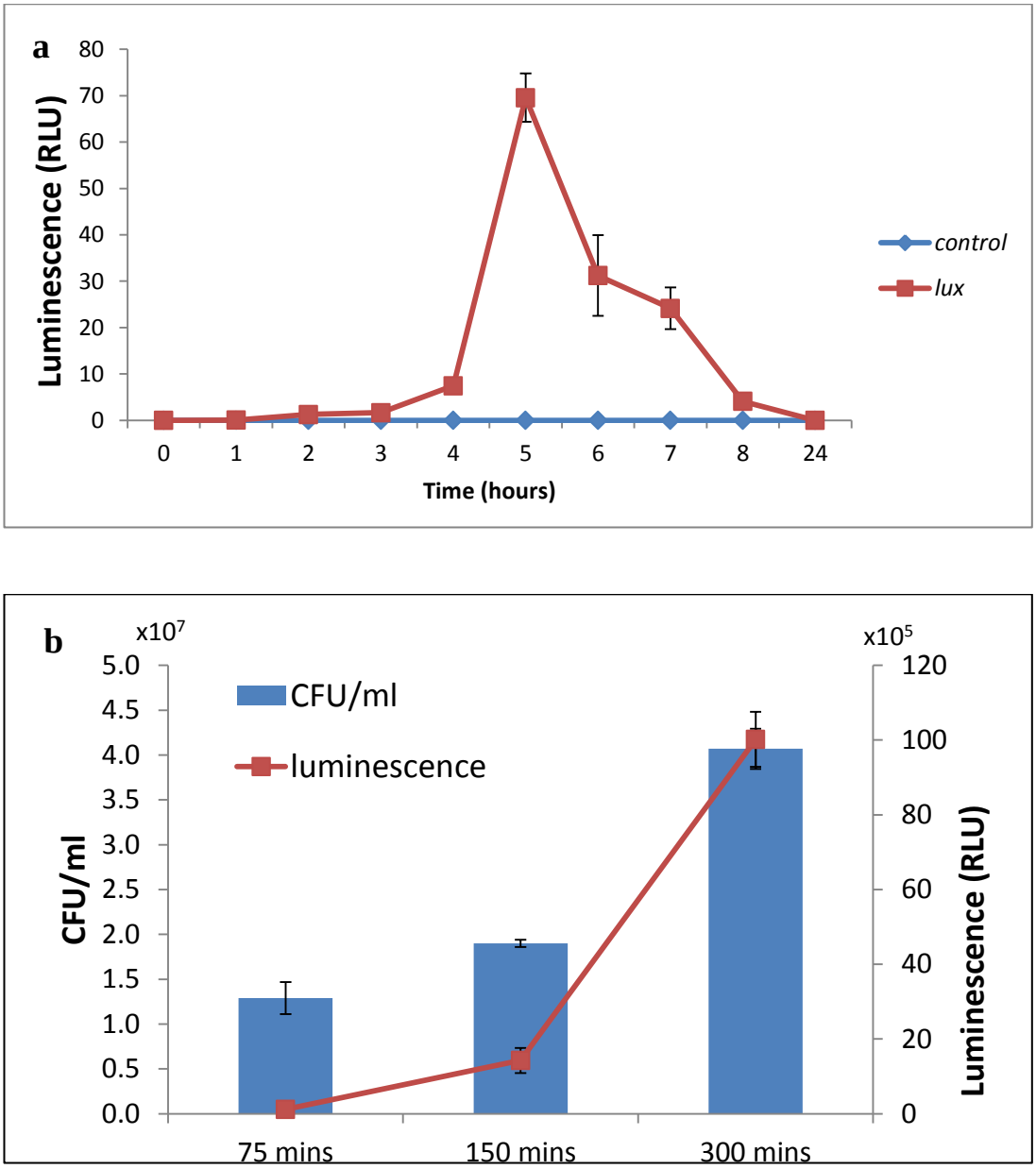


Figure 3.5: Production of bioluminescence from the *luxCDABE* operon.

E. coli cells transformed with the *lux* operon produced some luminescence which was measured at different time intervals (a).) Luminescence was shown to correlate with bacterial cell number (b). Error bars indicate standard errors between 3 biological replicates.

Since the luminescence of the strain carrying the *lux* construct diminished after 5 hr, growth (OD_{600}) was measured at different times in order to find out if the cells were still growing. The cell densities of both the *lux* strain and the control carrying an empty vector increased with time (Figure 3.6). An observation worth noting is that cell growth in the *lux* strain was compromised compared to the control strain after 2 hr of incubation until 24 hr. This is probably because of the extra metabolic burden placed on the lux strain to express the luciferase genes and emit luminescence.

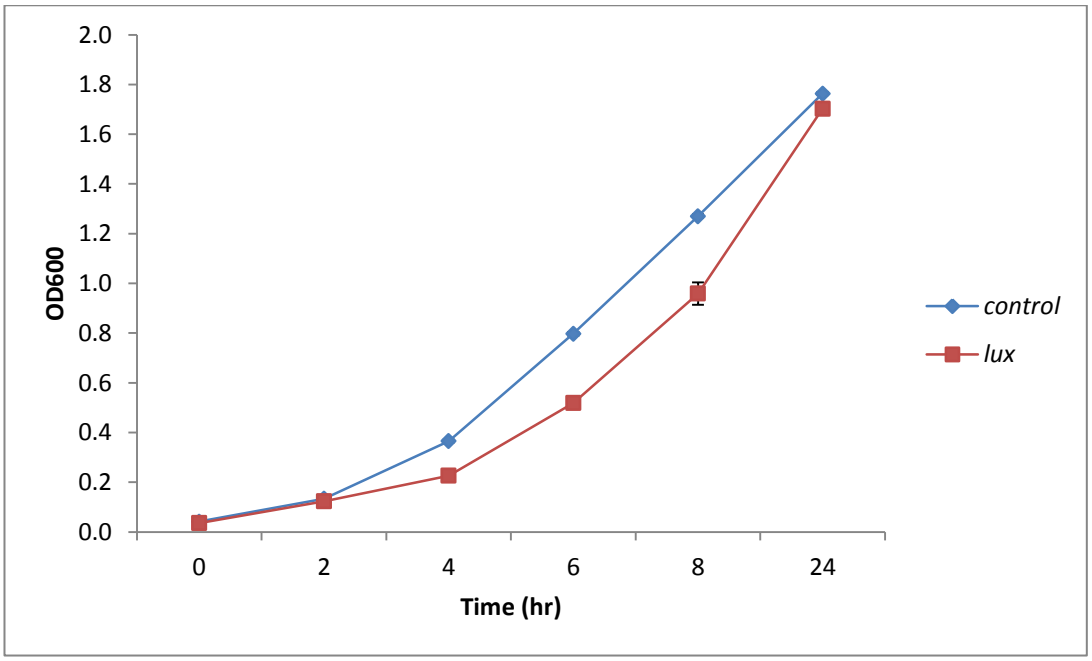


Figure 3.6: The effect of *luxCDABE* overexpression on the growth of *E. coli*.

Growth of cells overexpressing the *lux* operon was compromised during the period of bioluminescence production. Error bars show standard error of three biological replicates.

The effect of the various BioBricks in increasing organic solvent tolerance was studied with the bioluminescence assay. The *E. coli* cells, co-expressing a solvent tolerance gene and the *lux* operon, were grown in 4% ethanol and 0.5% *n*-butanol in M9 for 5 hr. The amount of luminescence produced by the cells per OD_{600} of the cultures was measured. This was divided by that of the cells grown in the absence of the solvent to determine the specific luminescence ratio. The higher the ratio, the higher the metabolic activity of the cell which indicates the viability of the cells even after exposure to the solvents. Results obtained with this assay were consistent with the other two assays with a few exceptions. The *phPFD*, *manXYZ* and *sodA* overexpressing strains exhibited a significant ($p<0.05$) improvement in ethanol tolerance (Figure 3.7a) consistent with the previous data. However, other strains such as *feoA* and *marA* which had shown ethanol tolerance in the other assays did not appear tolerant to ethanol when the bioluminescence assay was used. During *n*-butanol challenge *phPFD*, *manXYZ*, *glpC* and *marA* significantly ($p<0.05$) improved tolerance (Figure 3.7b). Atf1 which had been shown to increase tolerance to *n*-butanol in the previous assays did not increase *n*-butanol tolerance with this assay.

The library was also screened for tolerance to furfural, an inhibitor produced during biomass pre-treatment, and a different organic solvent which is not an alcohol, acetone. Interestingly, none of the BioBricks protected the cells from furfural toxicity (Figure 3.7c). Strains overexpressing *glpC* and *feoA* were more sensitive to 15 mM furfural compared to the control strain. Also, following an initial screening of all the constructs in 3% acetone after 24 hr incubation in LB, five of the strains that had relatively high cell densities were tested with the luminescence assay. The strains tested, *phPFD*, *dps*, *rcsA*, *atf1* and *katG* did not show increased tolerance to acetone (Figure 3.7d).

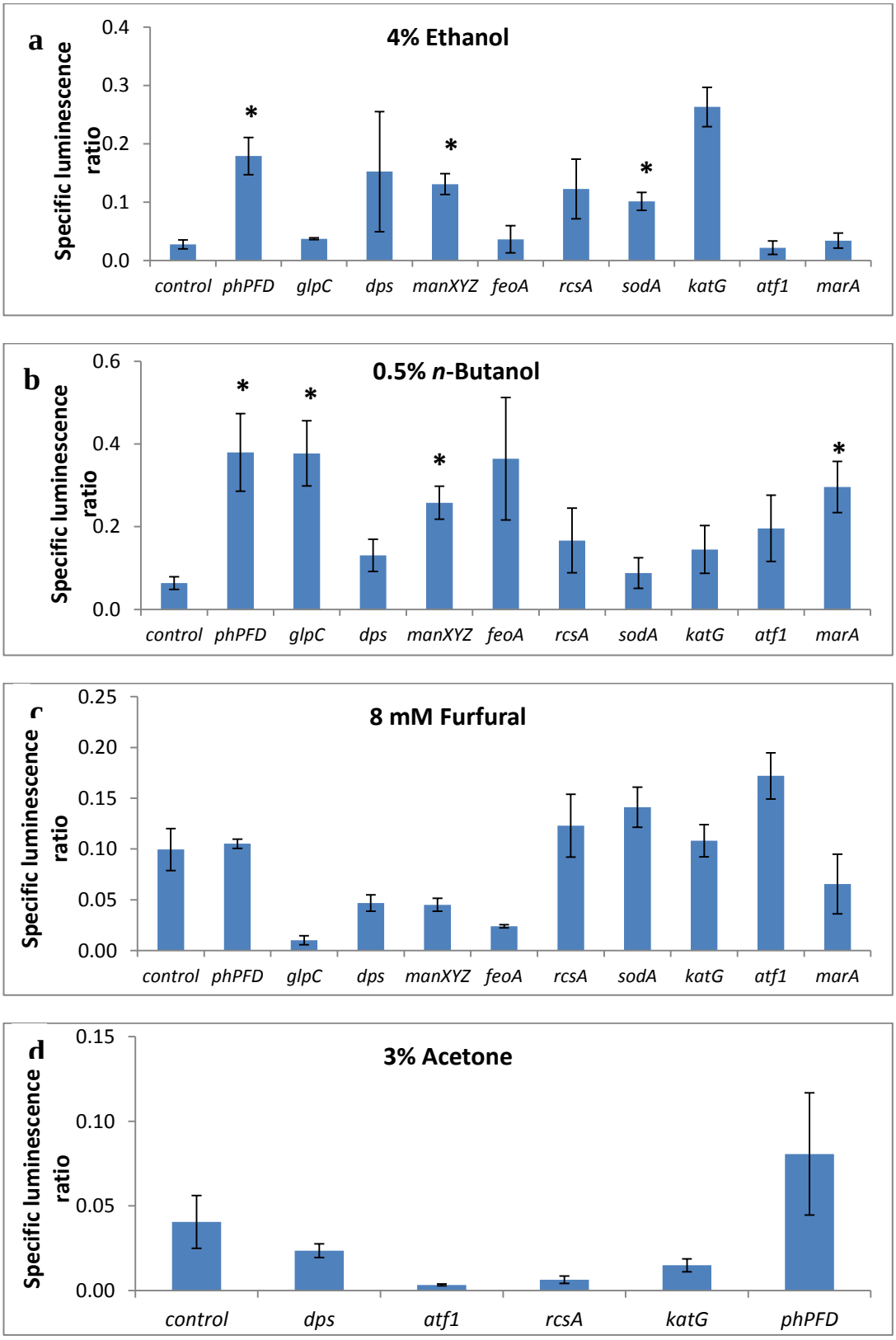


Figure 3.7: Assaying solvent tolerance by measuring luciferase activity.

Tolerance was measured in the presence of a) 4% ethanol b) 0.5% *n*-butanol c) 8 mM fufural and d) 3% acetone. Error bars indicate standard errors between 3 biological replicates. Asterisks indicate statistical significance ($p<0.05$).

3.2.4 Growth kinetics of top performing strains

In order to further study the effect of ethanol and *n*-butanol on tolerant strains, a time-course growth assay was performed with the best performing strains, *phPFD*, *glpC, manXYZ* and *marA* to determine their growth relative to the control in the presence of these alcohols at different time points. Growth was determined as a measure of increase in biomass (OD_{595}). The top strains were those that increased ethanol or *n*-butanol tolerance in two or three out of the three assays used for the screening. Since the *E. coli* JM109 strain used for the above experiments is not the most ideal *E. coli* strain for producing toxic compounds industrially, the parent strain MG1655 was transformed with these constructs and tested it to determine the ability of the top constructs to increase ethanol and *n*-butanol tolerance in a different *E. coli* background. The top strains were also screened in concentrations higher than previously tested in this study. At lower concentrations of ethanol, the cell growth of the different strains was identical to control strain (Figures 3.8, 3.9 and 3.10). However, at higher concentrations *phPFD* and *marA* grew better than the control strain (Figure 3.11). At high ethanol concentrations, *marA* shows a prolonged lag phase but eventually outgrows the control strain.

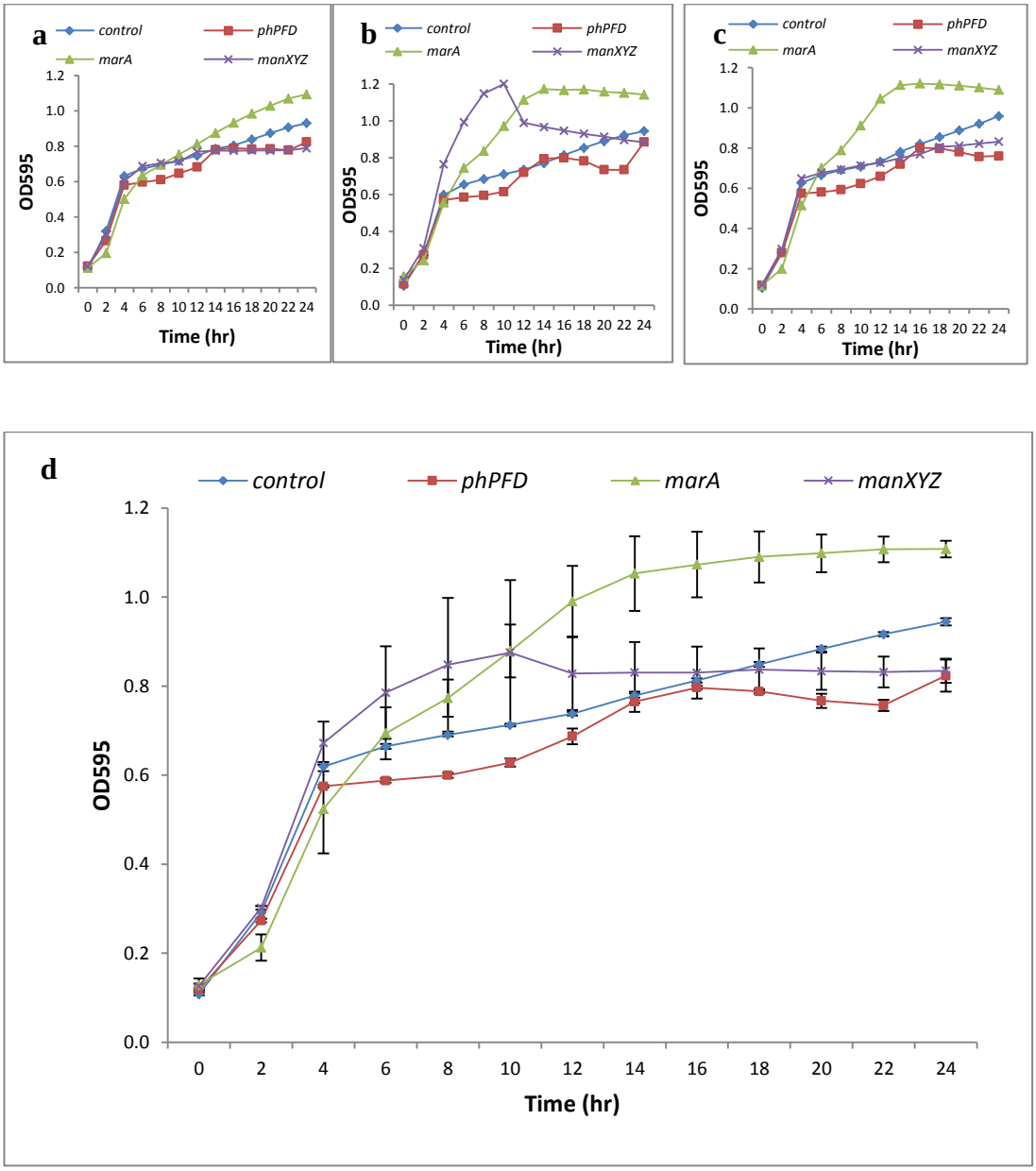


Figure 3.8: Time-course experiments of top performing strains in cultures containing 0% ethanol.

E. coli MG1655 cells carrying different solvent tolerance genes were grown in LB in the absence of ethanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

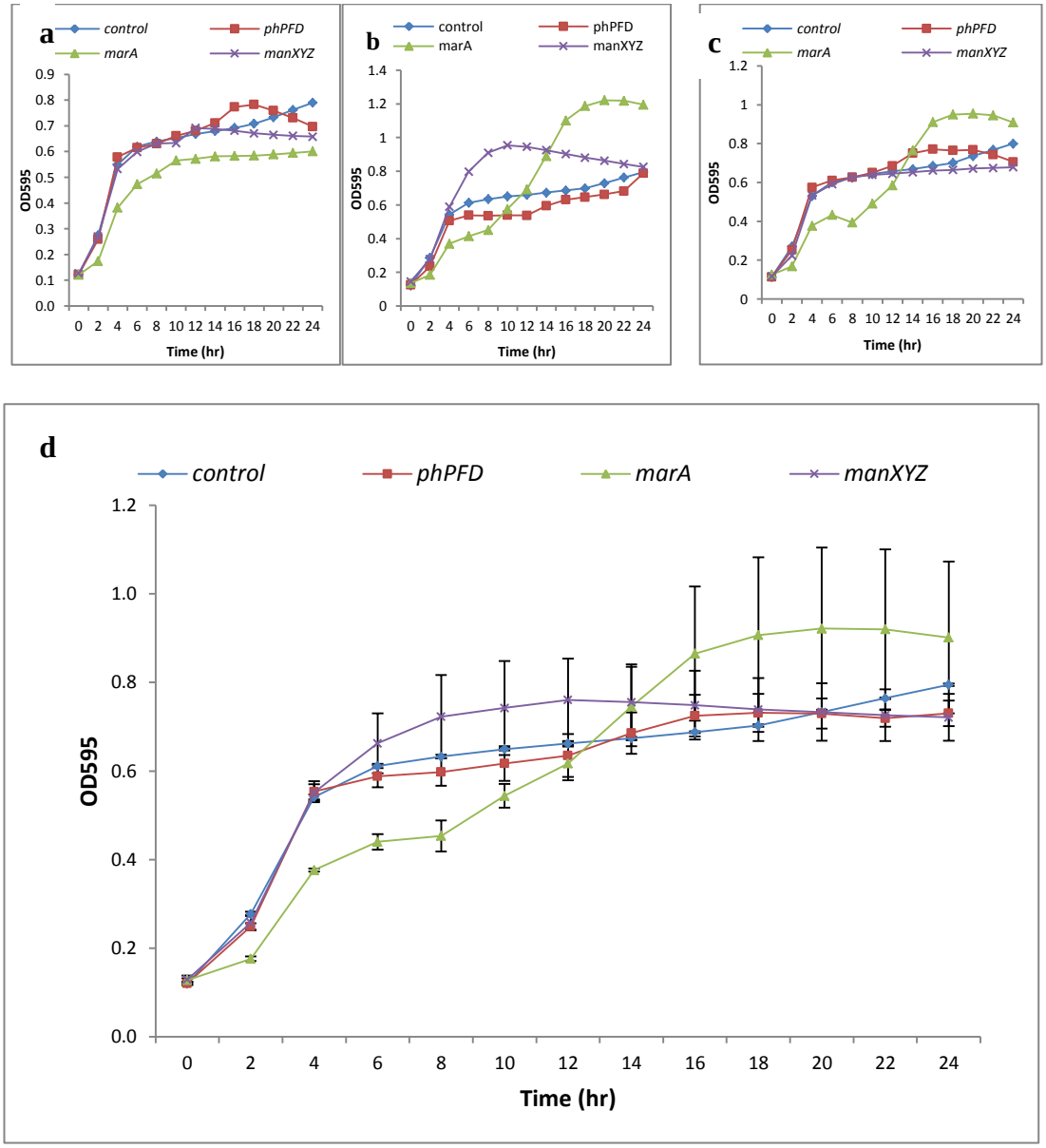


Figure 3.9: Time-course experiments of top performing strains in cultures containing 2% ethanol.

E. coli MG1655 cells carrying different solvent tolerance genes were grown in LB containing 2% ethanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

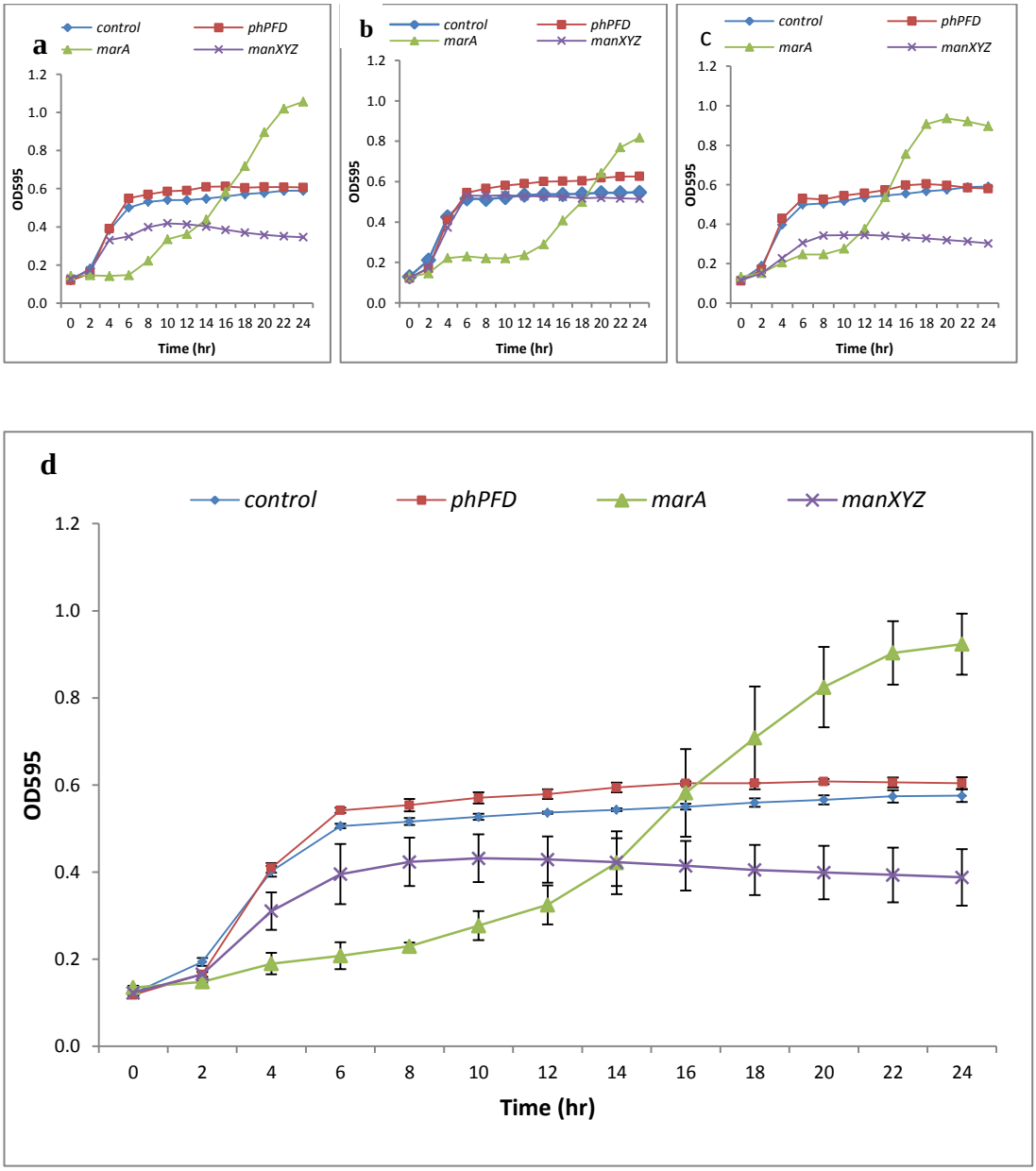


Figure 3.10: Time-course experiments of top performing strains in cultures containing 4% ethanol.

E. coli MG1655 cells carrying different solvent tolerance genes were grown in LB containing 4% ethanol and growth (OD_{595}) was measured at different time points. The *marA* strain shows an improved growth even though it has a long lag phase. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

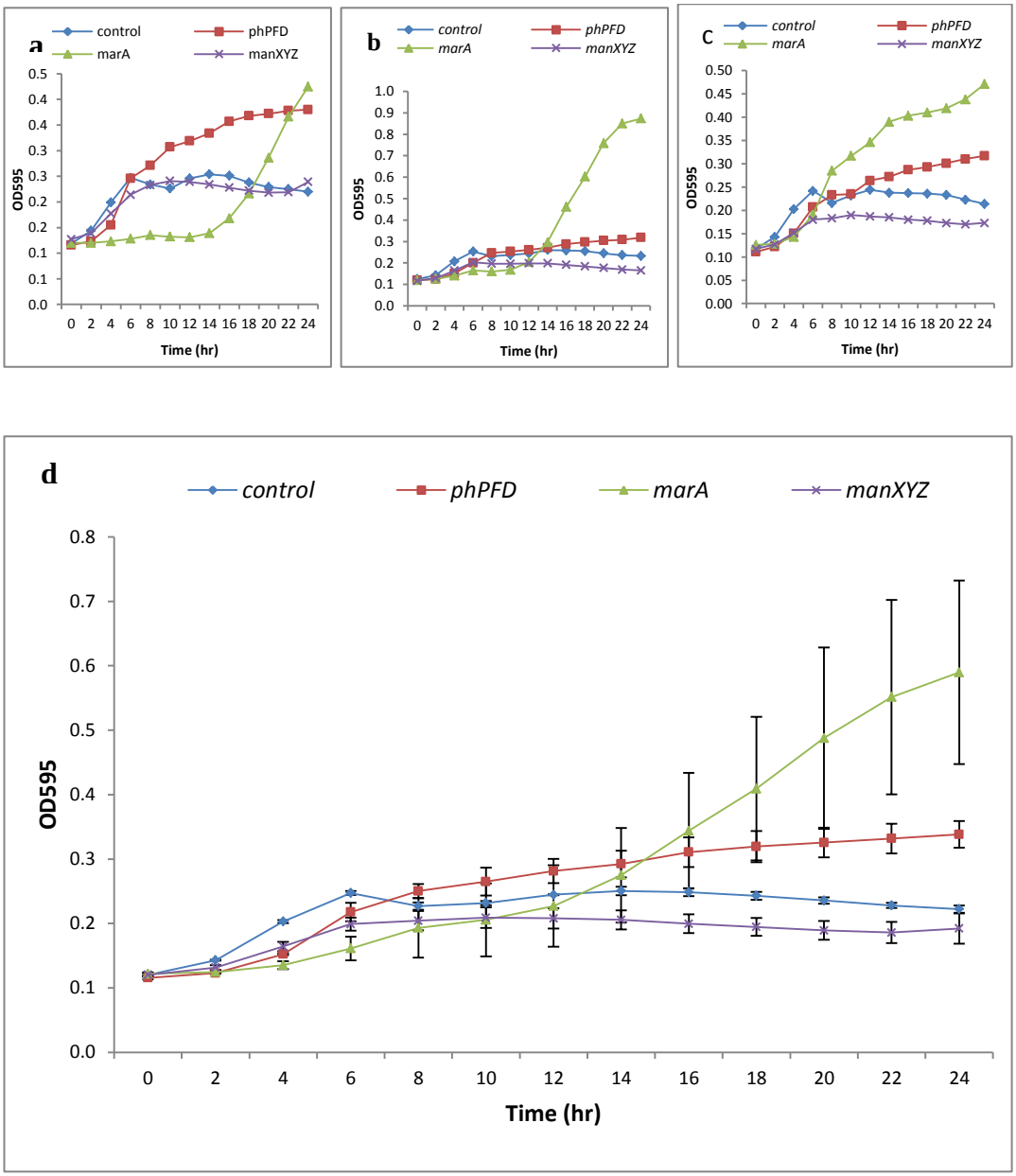
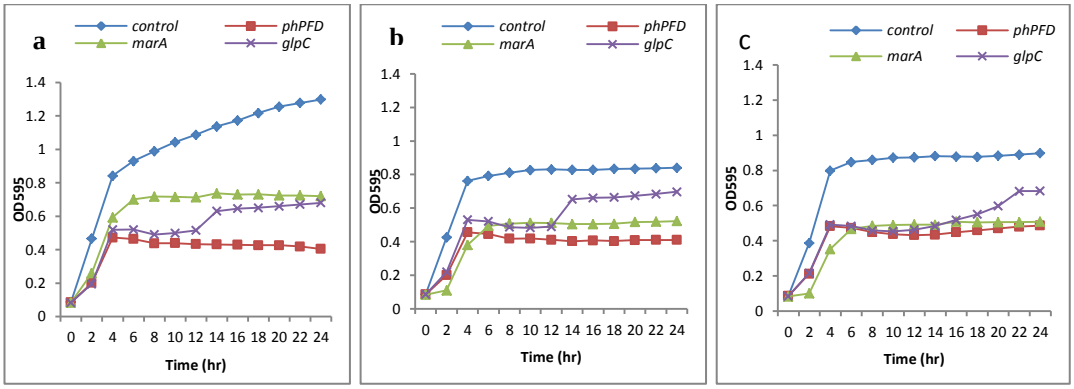


Figure 3.11: Time-course experiments of top performing strains in cultures containing 6% ethanol.

E. coli MG1655 cells carrying different solvent tolerance genes were grown in LB containing 6% ethanol and growth (OD_{595}) was measured at different time points. The *marA* and *phPFD* strains grow better than the control strain at this concentration of ethanol. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

The effect of *n*-butanol on the *E. coli* MG1655 strain overexpressing the top butanol tolerance genes was also examined at different time points and at different concentrations. At low concentrations of *n*-butanol, the control strain grew better than the other strains (Figure 3.12). Even though the control strain started off well at 0% *n*-butanol, the effect of increasing concentrations of *n*-butanol on the cells were more distinct than on the other strains. For instance, the average final absorbance (OD_{595}) attained by the control strain at 0.5% *n*-butanol dropped by 0.493 units when compared with the average final absorbance (OD_{595}) at 0% *n*-butanol. For *phPFD, marA* and *glpC*, the average final absorbance (OD_{595}) at 0% *n*-butanol dropped by 0.097, 0.198 and 0.288 units respectively at 0.5% showing a higher resistance than the control strain. Also, at 0.7% and 0.9%, the top strains performed better in growth compared to the control strain (Figures 3.14 and 3.15). At 1.1% *n*-butanol, there was no observable growth in all the strains tested (Figure 3.16). Similar to the ethanol experiment, the *marA* strain had a prolonged lag phase when *n*-butanol was added to the culture.



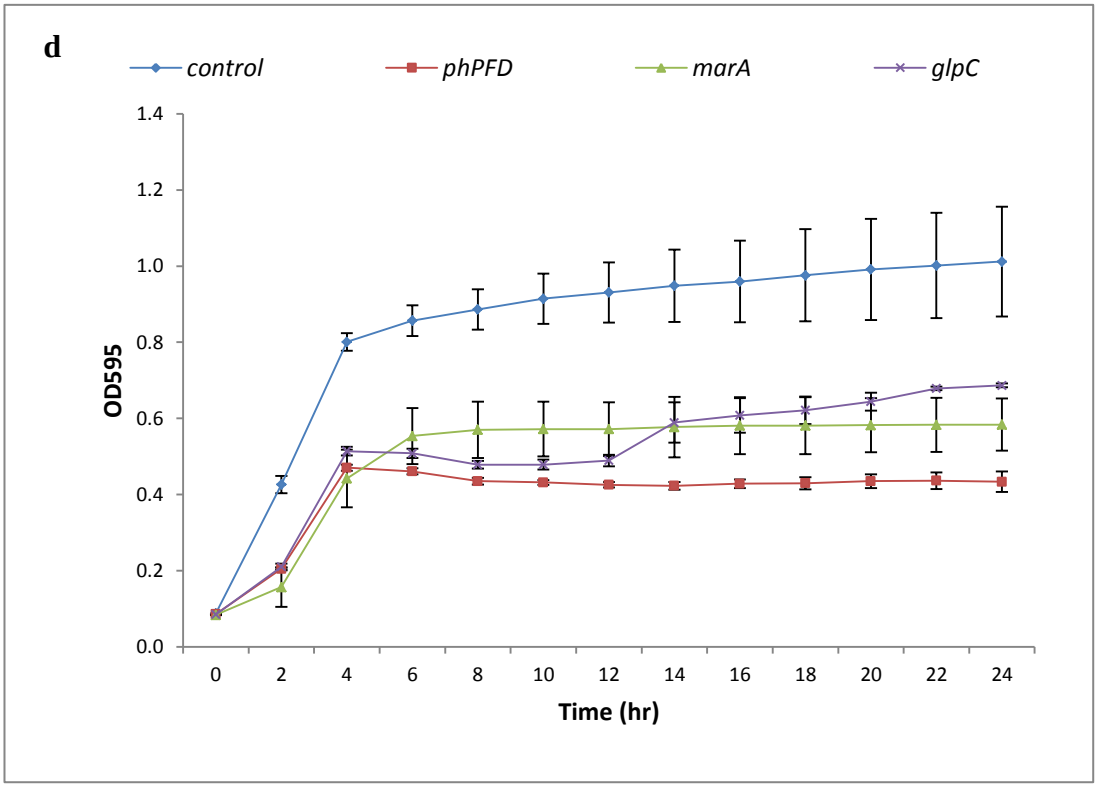


Figure 3.12: Time-course experiments of top performing strains in cultures containing 0% *n*-butanol.

E. coli MG1655 cells carrying different solvent tolerance genes were grown in LB without the addition of *n*-butanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

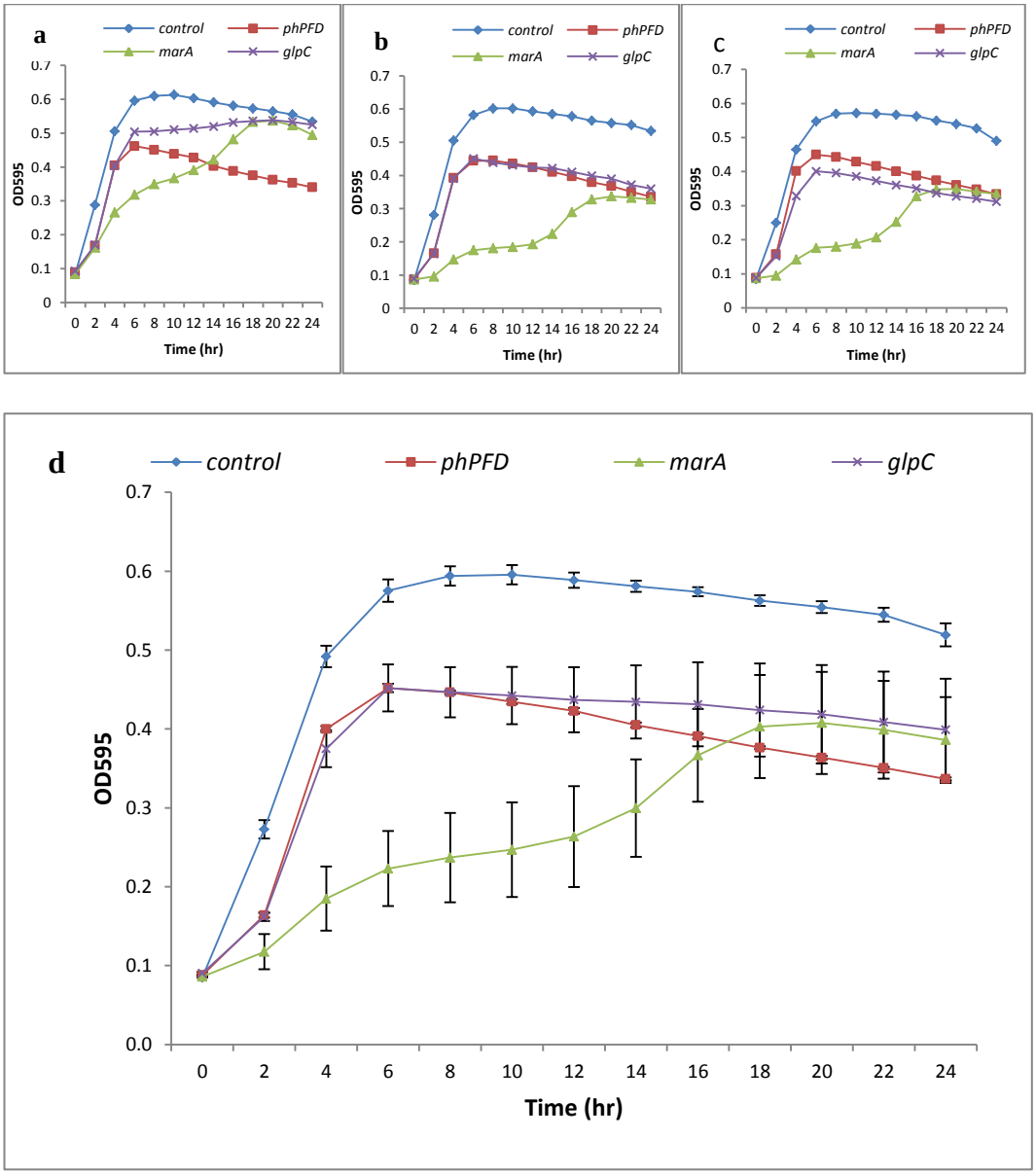


Figure 3.13: Time-course experiments of top performing strains in cultures containing 0.5% *n*-butanol.

E. coli MG1655 cells carrying different solvent tolerance genes were grown in LB containing 0.5% *n*-butanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

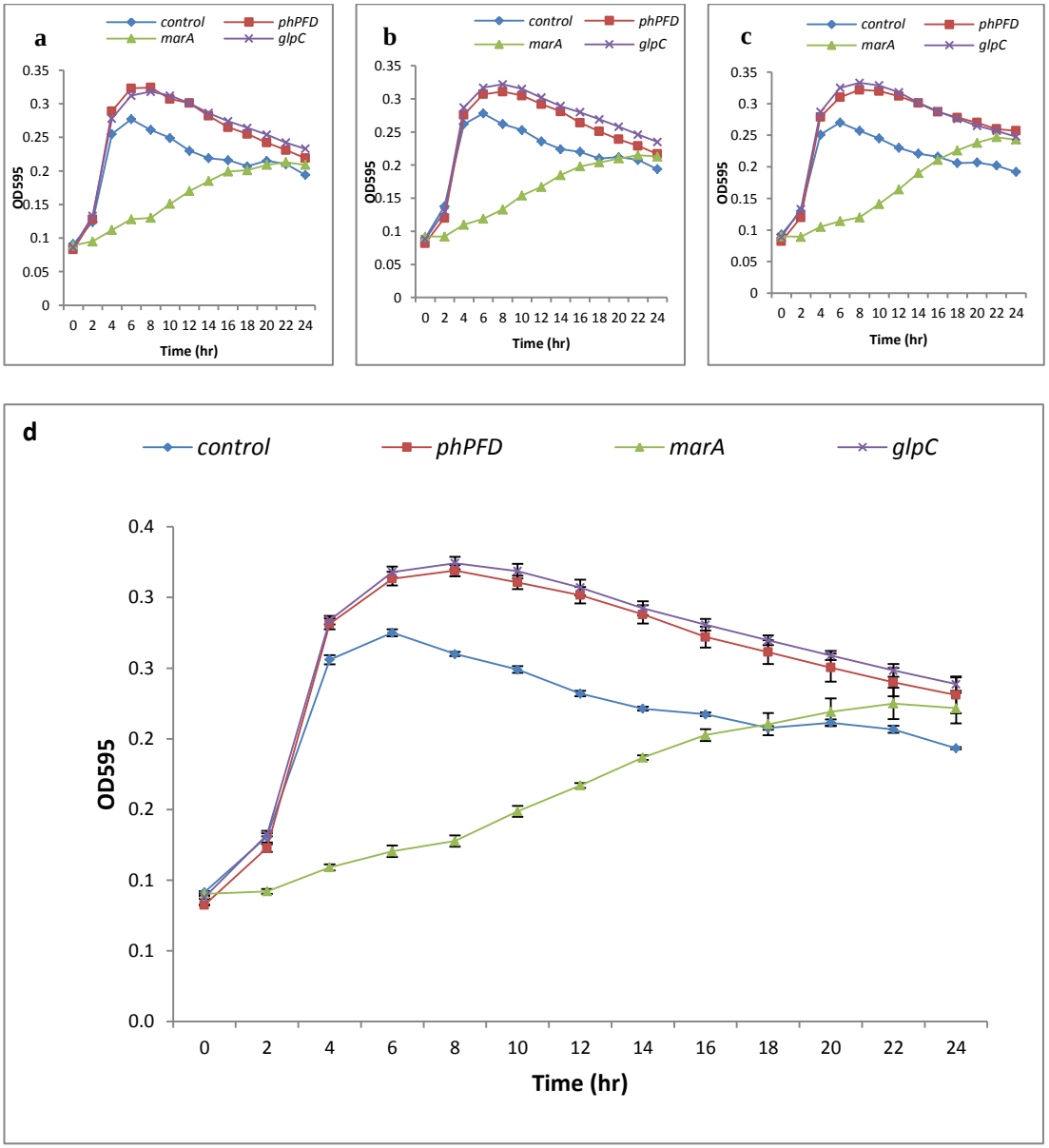


Figure 3.14: Time-course experiments of top performing strains in cultures containing 0.7% *n*-butanol.

E. coli MG1655 cells carrying different solvent tolerance genes were grown in LB containing 0.7% *n*-butanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

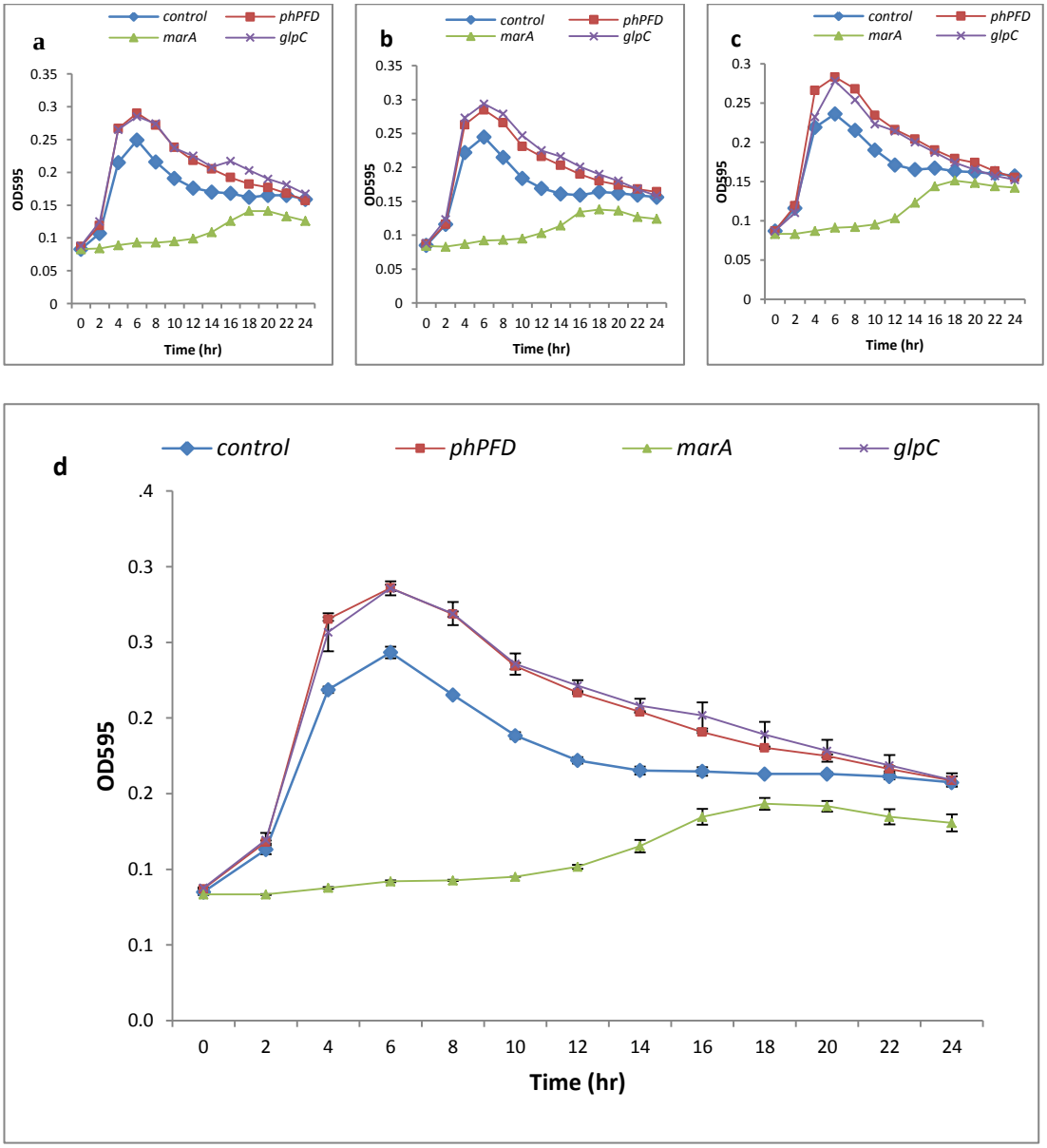


Figure 3.15: Time-course experiments of top performing strains in cultures containing 0.9% *n*-butanol.

E. coli MG1655 cells carrying different solvent tolerance genes were grown in LB containing 0.9% *n*-butanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

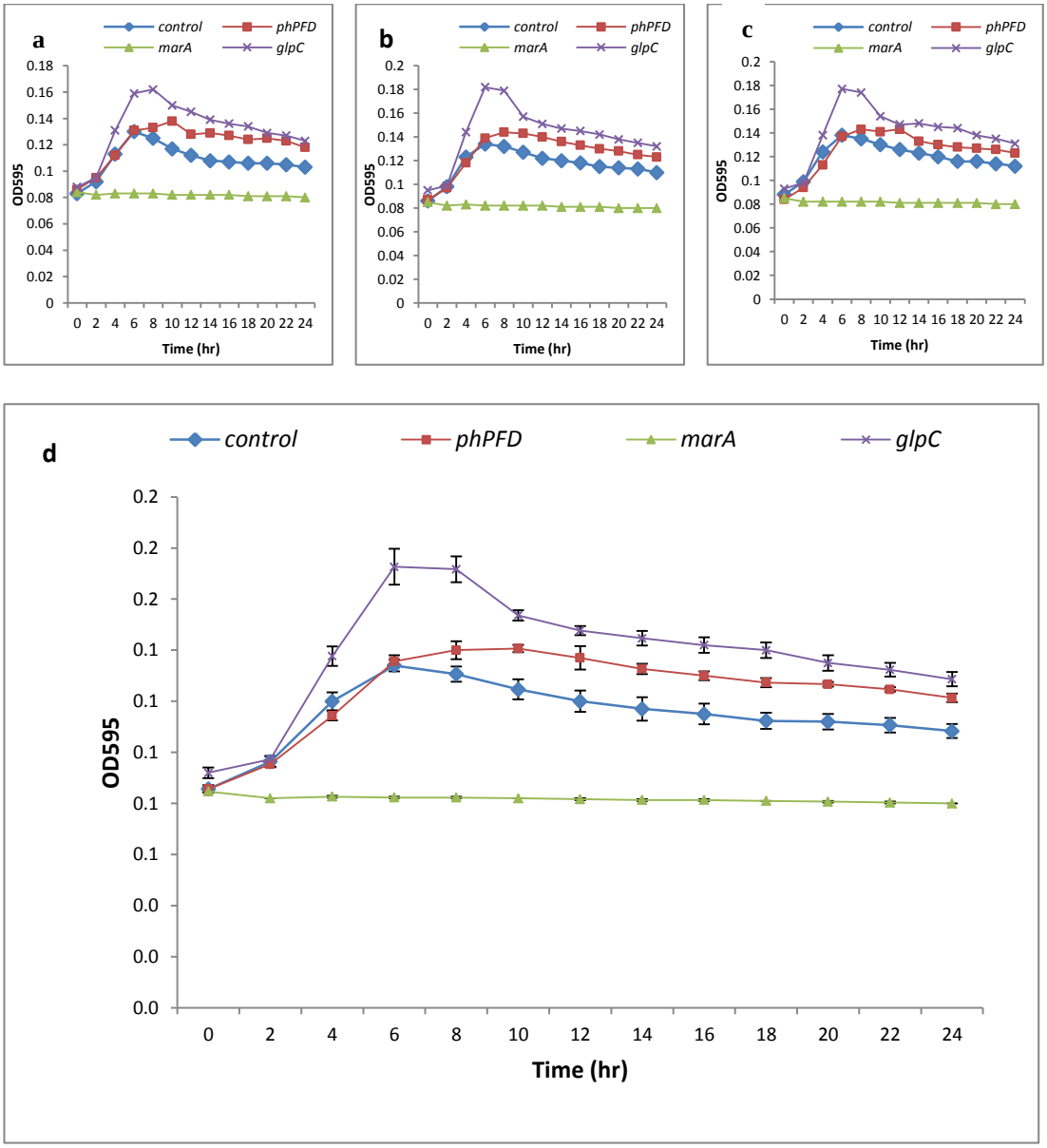


Figure 3.16: Time-course experiments of top performing strains in cultures containing 1.1% *n*-butanol.

E. coli MG1655 cells carrying different solvent tolerance genes were grown in LB containing 1.1% *n*-butanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

3.2.5 Effect of *groESL* on ethanol and *n*-butanol tolerance

Looking at the results obtained so far in this chapter, *phPFD*, a heterologous gene originally cloned from *Pyrococcus horikoshi* (Okochi *et al*., 2008) consistently increased tolerance to ethanol and *n*-butanol when overexpressed in *E. coli*. Therefore, the effect of a native *E. coli* protein chaperone, *groESL*, on ethanol and *n*-butanol tolerance was determined and compared to *phPFD*. *groESL* was chosen because it is one of the principal molecular chaperones in *E. coli* and has been shown to increase *E. coli* tolerance to different alcohols (Zingaro and Papoutsakis, 2012; Zingaro and Papoutsakis, 2013). The *groESL* genes were cloned from *E. coli* MG1655 and overexpressed in pSB1C3 under the control of the *lac* promoter. The effect of low expression of the genes on alcohol tolerance was determined by placing the construct on a low copy plasmid, pSB4K5, under the control of the *lac* promoter. The effect of various concentrations of ethanol and *n*-butanol on strains overexpressing *groESL* was studied at different time points. When overexpressed on pSB1C3, phPFD was able to increase tolerance to both ethanol (Figures 3.17 - 3.21) and *n*-butanol (Figures 3.22 – 3.26) in *E. coli* whereas *groESL* showed no improvement in tolerance to these alcohols. The growth of the *groESL* strain was comparable to the control strain.

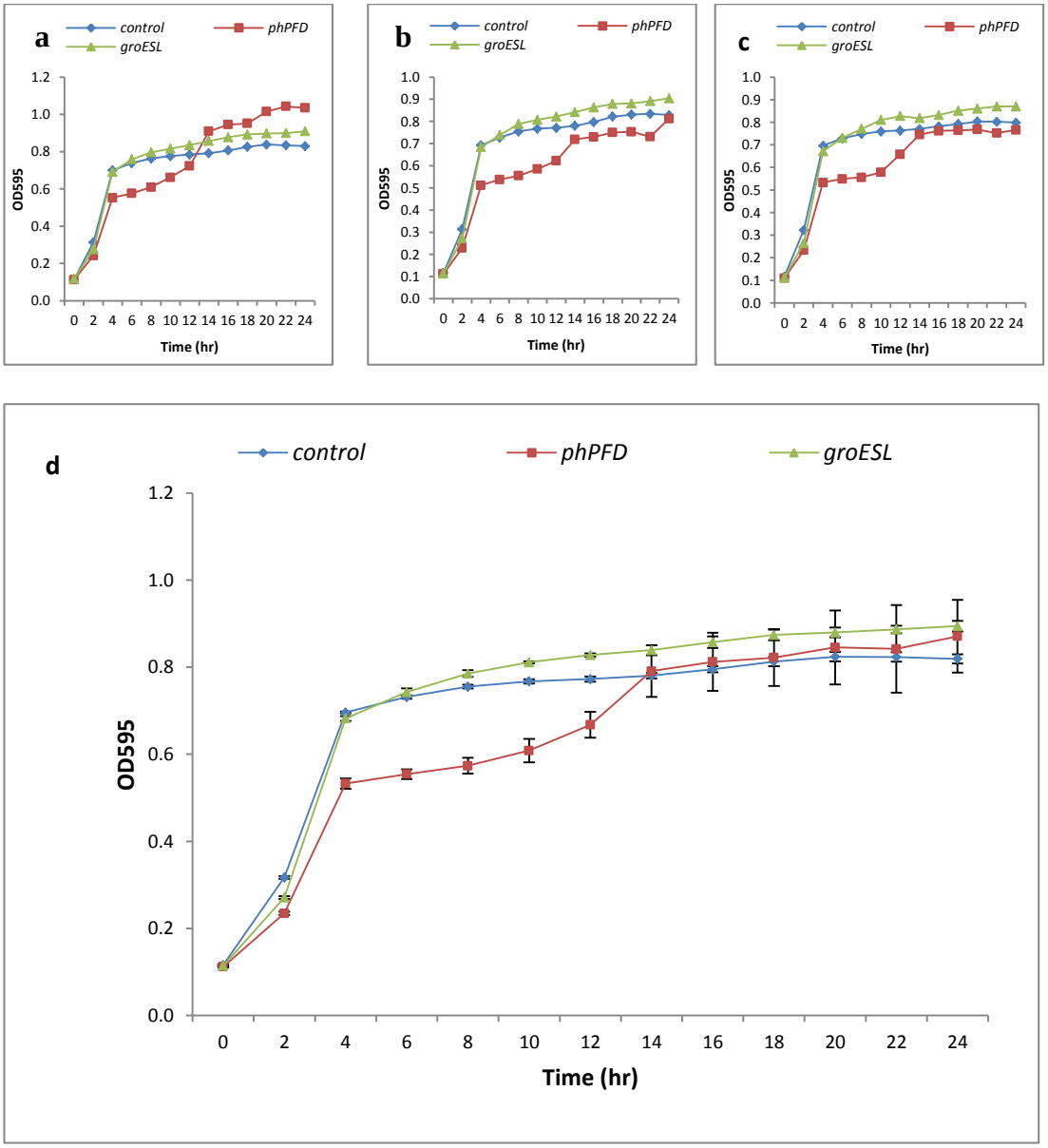


Figure 3.17: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB1C3) in cultures containing 0% ethanol.

E. coli MG1655 cells carrying different protein chaperones on a high copy number plasmid were grown in LB containing no ethanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

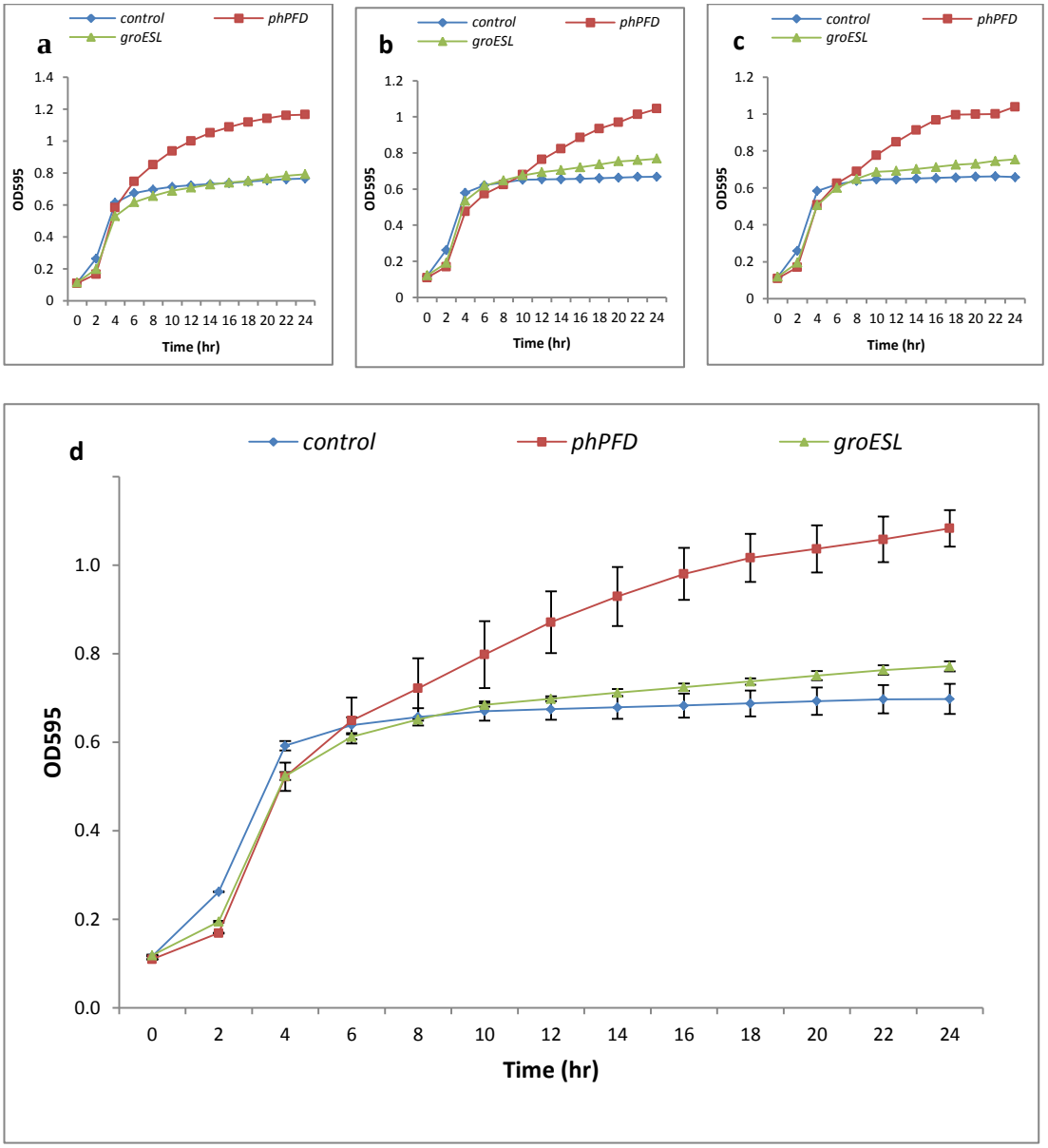


Figure 3.18: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB1C3) in cultures containing 2% ethanol.

E. coli MG1655 cells carrying different protein chaperones on a high copy number plasmid were grown in LB containing 2% ethanol and growth (OD_{595}) was measured at different time points. The *phPFD* strain grows better than the control strain at this concentration of ethanol. The *groESL* strain, however, shows a growth pattern similar to the control strain. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

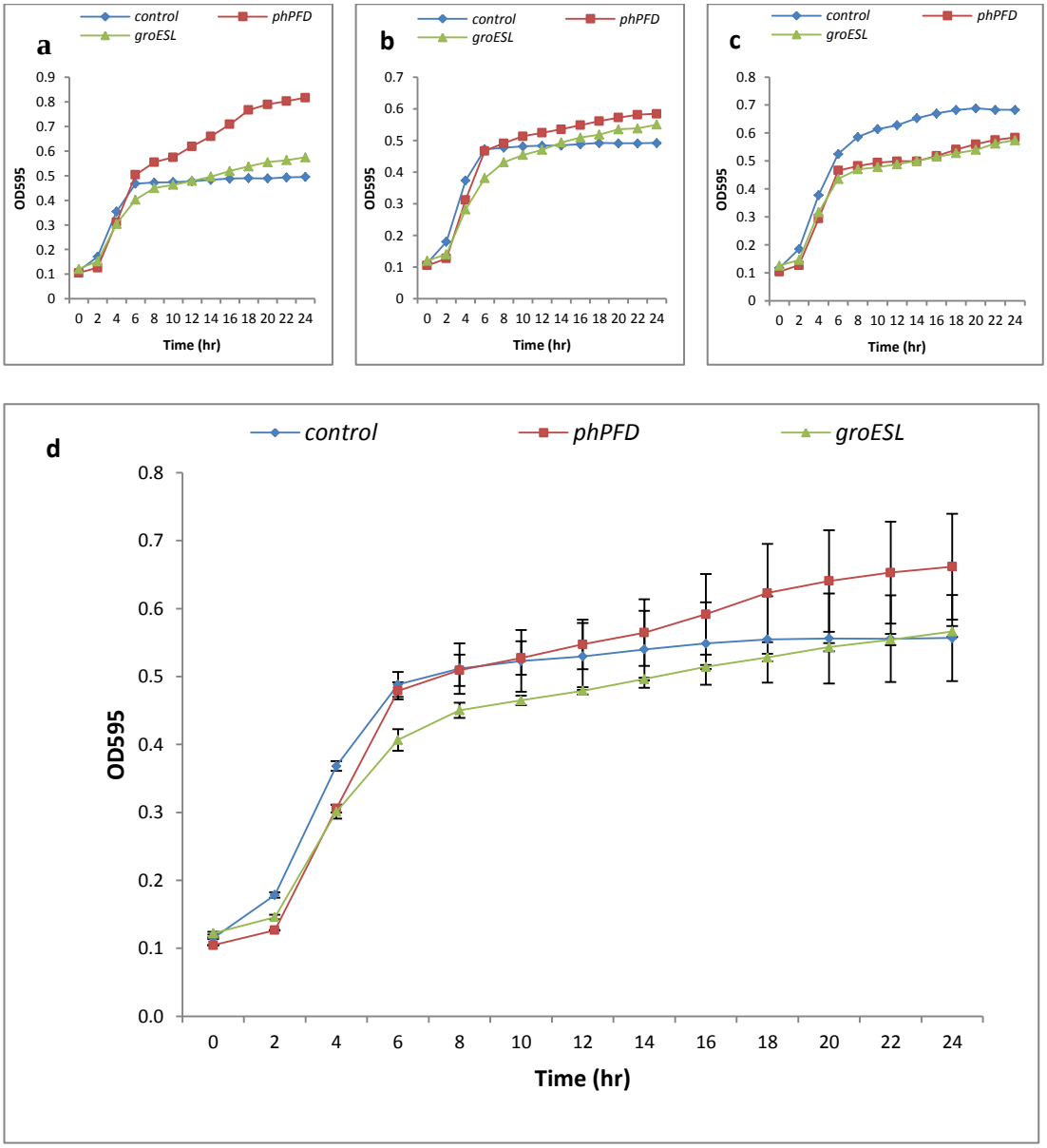


Figure 3.19: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB1C3) in cultures containing 4% ethanol.

E. coli MG1655 cells carrying different protein chaperones on a high copy number plasmid were grown in LB containing 4% ethanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

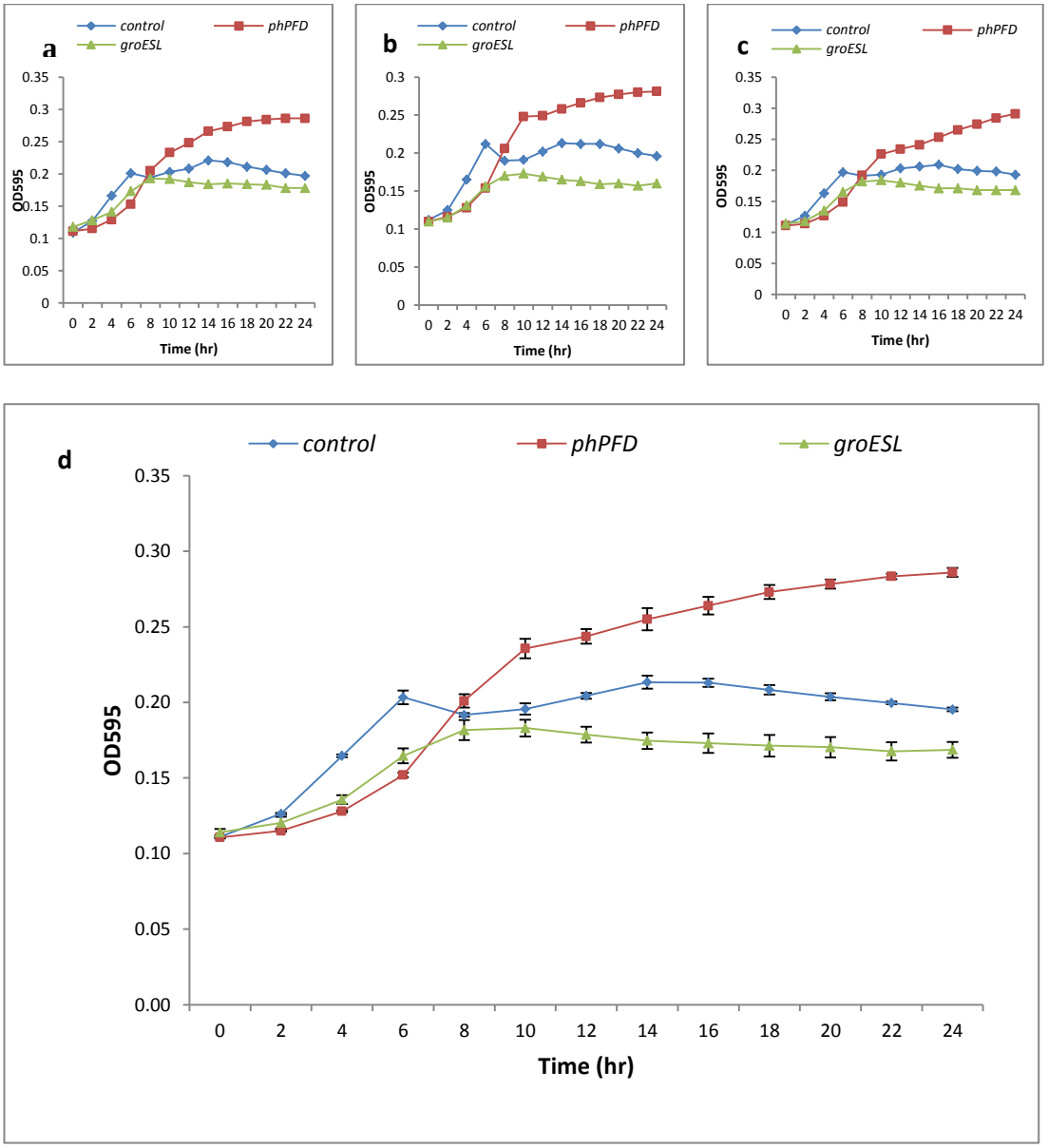


Figure 3.20: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB1C3) in cultures containing 6% ethanol.

E. coli MG1655 cells carrying different protein chaperones on a high copy number plasmid were grown in LB containing 6% ethanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

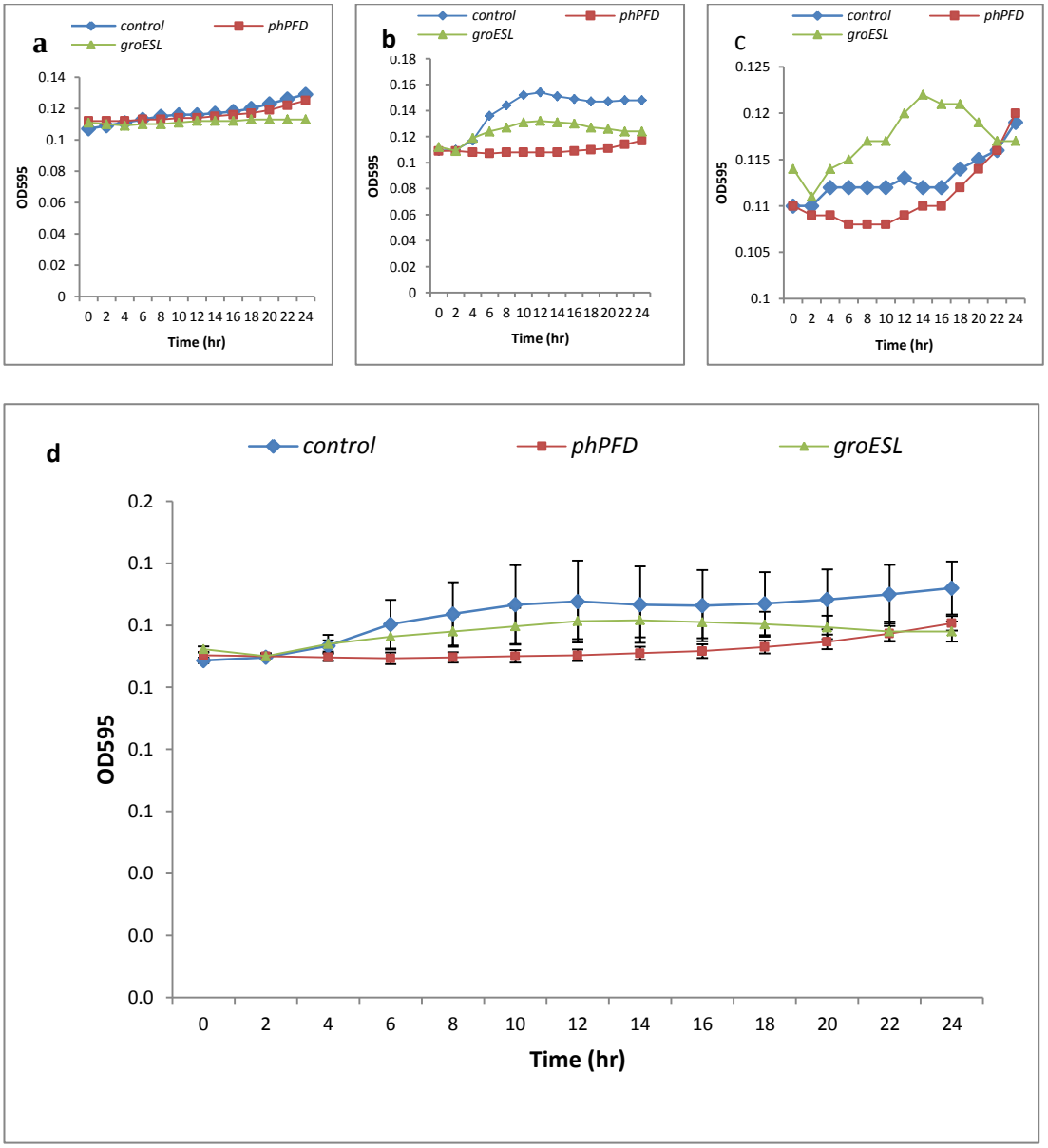


Figure 3.21: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB1C3) in cultures containing 8% ethanol.

E. coli MG1655 cells carrying different protein chaperones on a high copy number plasmid were grown in LB containing 8% ethanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

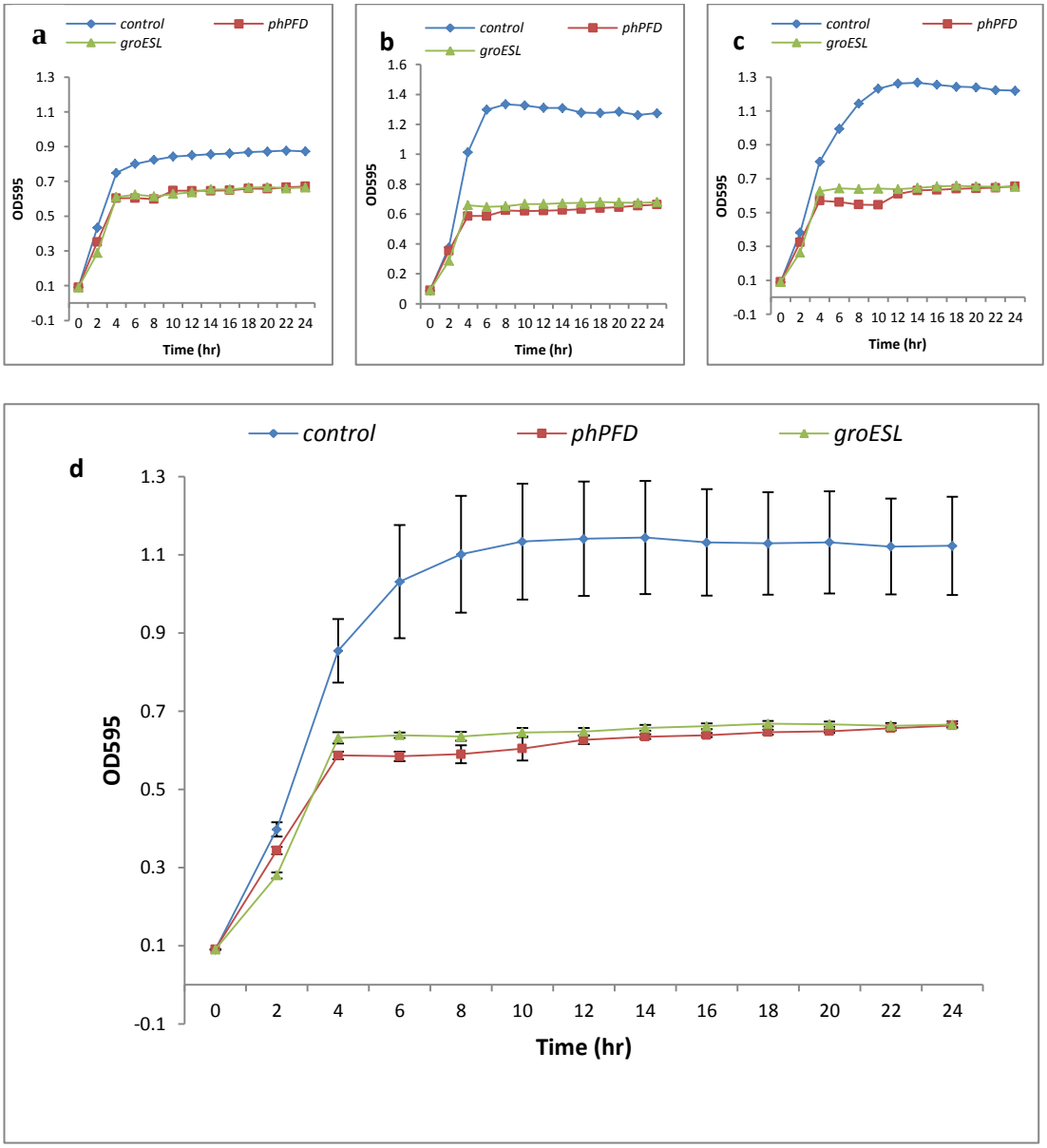


Figure 3.22: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB1C3) in cultures containing 0% *n*-butanol.

E. coli MG1655 cells carrying different protein chaperones on a high copy number plasmid were grown in LB containing no *n*-butanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

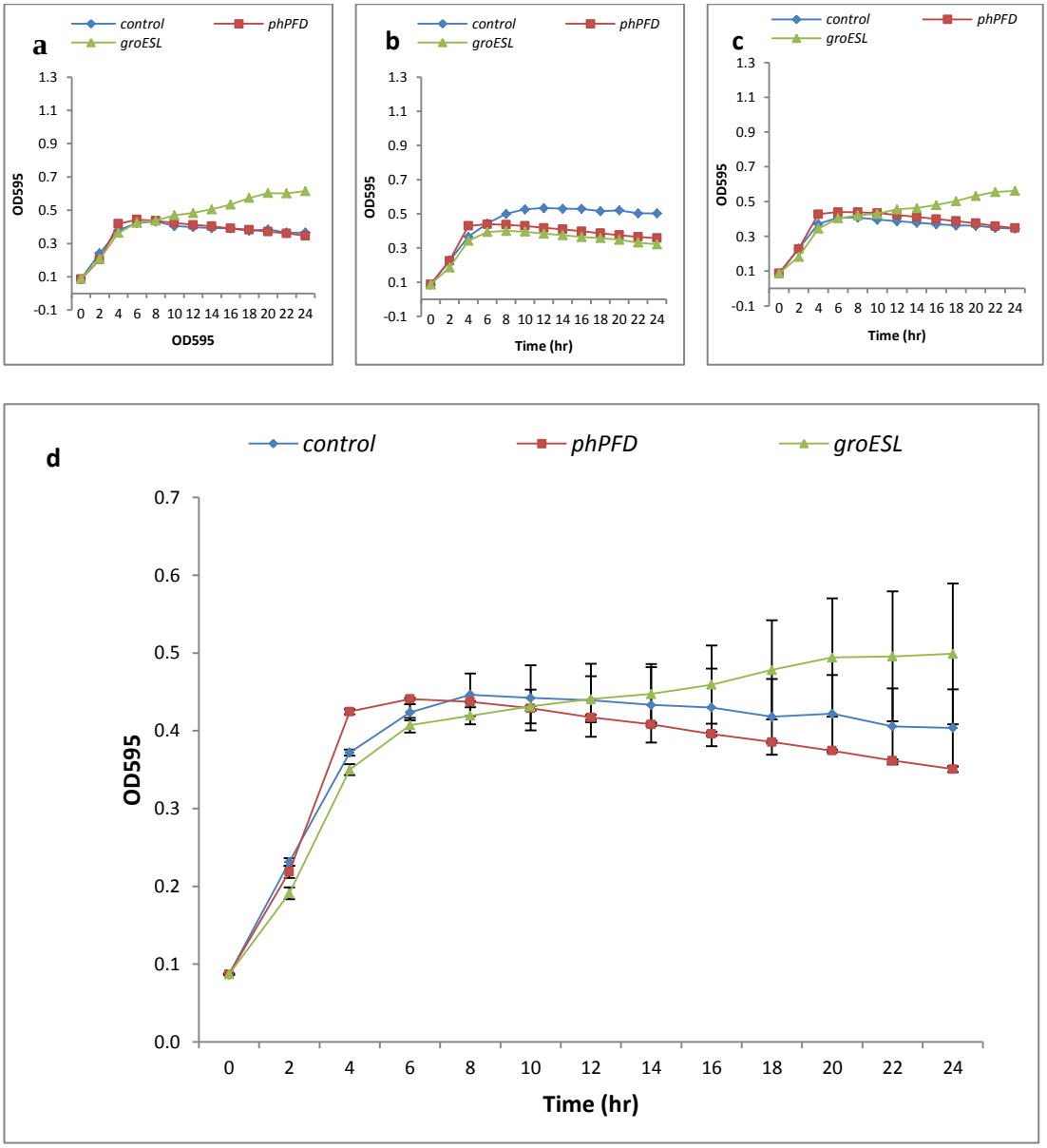


Figure 3.23: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB1C3) in cultures containing 0.5% *n*-butanol.

E. coli MG1655 cells carrying different protein chaperones on a high copy number plasmid were grown in LB containing 0.5% *n*-butanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

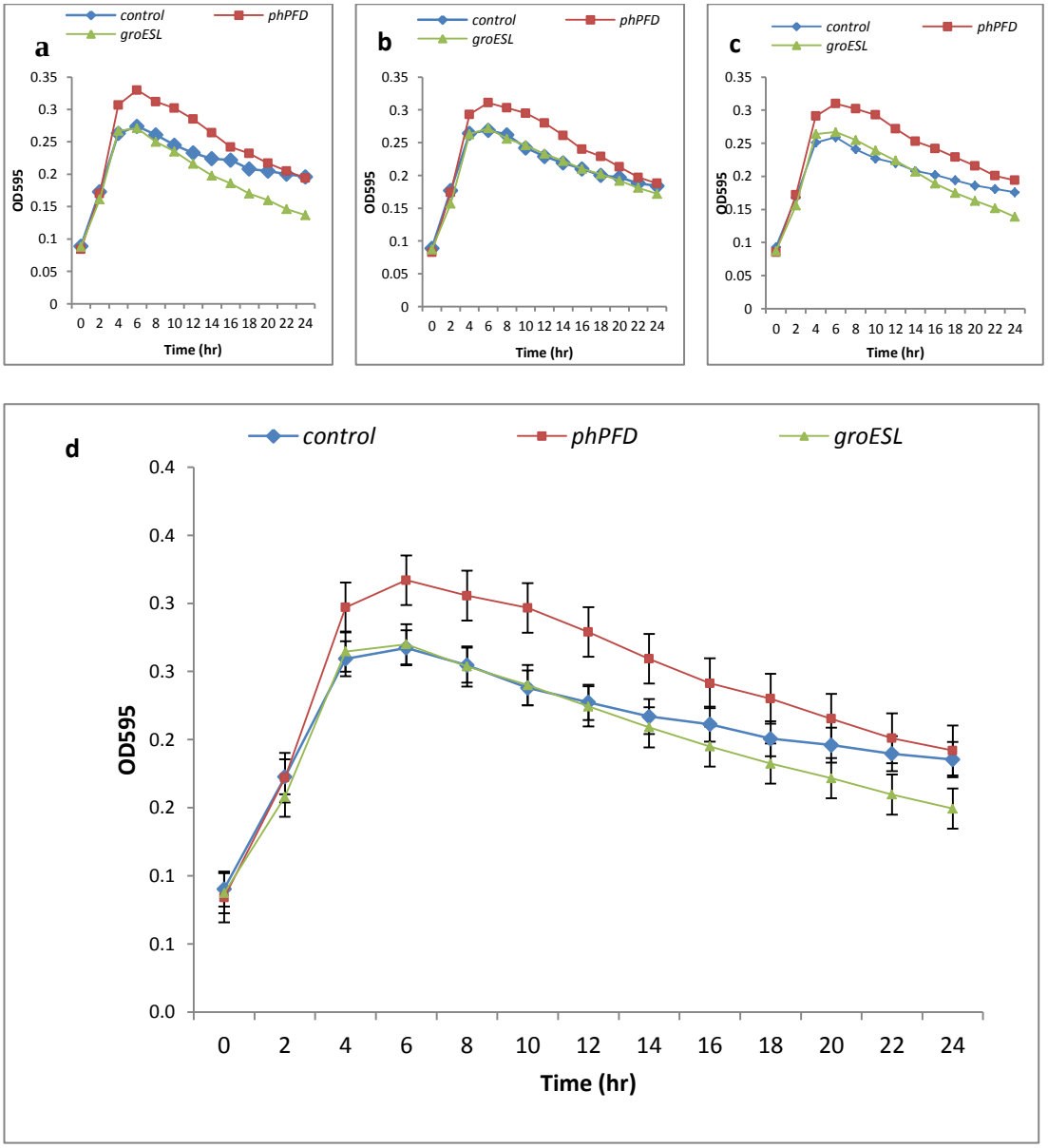


Figure 3.24: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB1C3) in cultures containing 0.7% *n*-butanol.

E. coli MG1655 cells carrying different protein chaperones on a high copy number plasmid were grown in LB containing 0.7% *n*-butanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

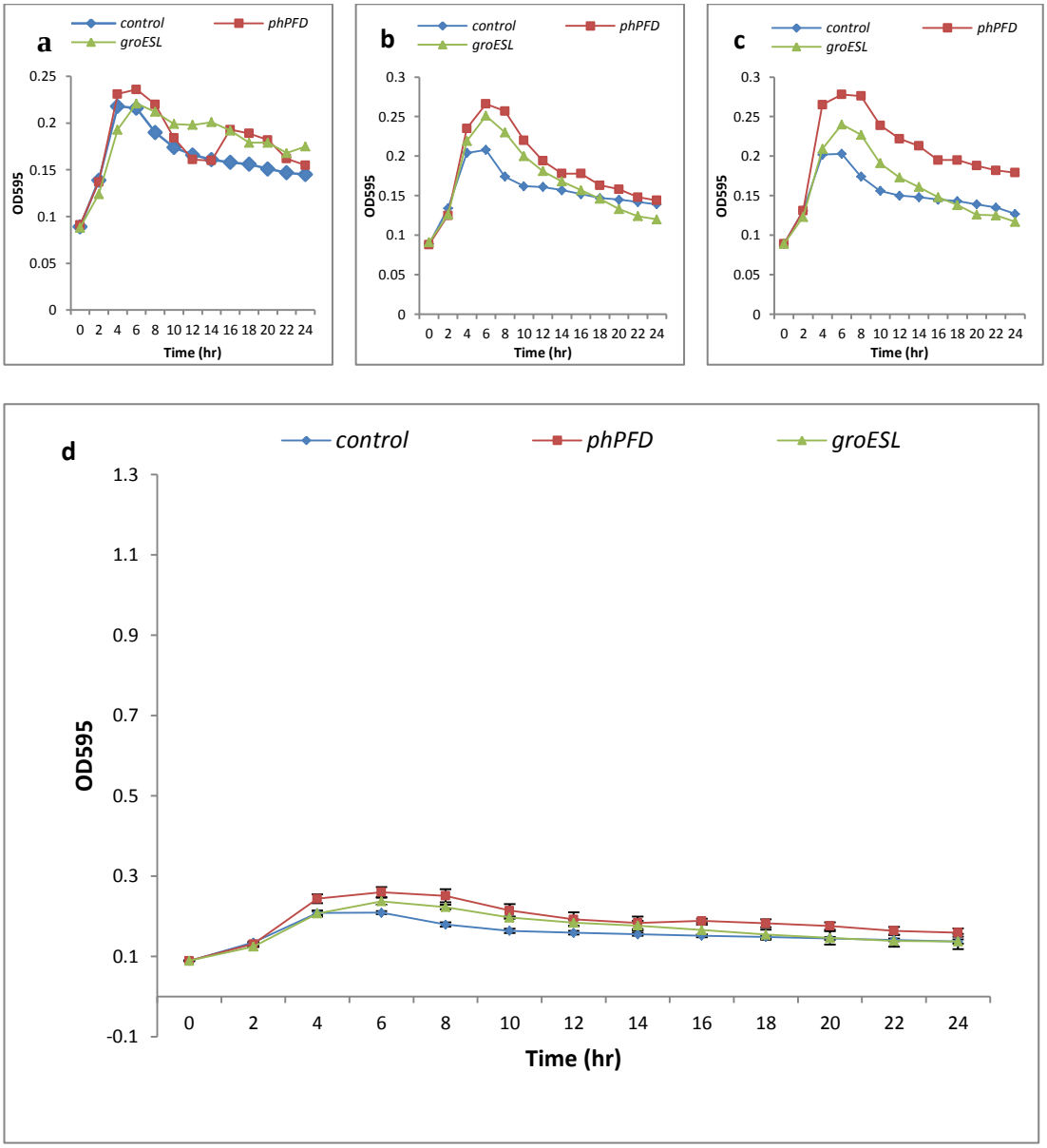


Figure 3.25: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB1C3) in cultures containing 0.9% *n*-butanol.

E. coli MG1655 cells carrying different protein chaperones on a high copy number plasmid were grown in LB containing 0.9% *n*-butanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

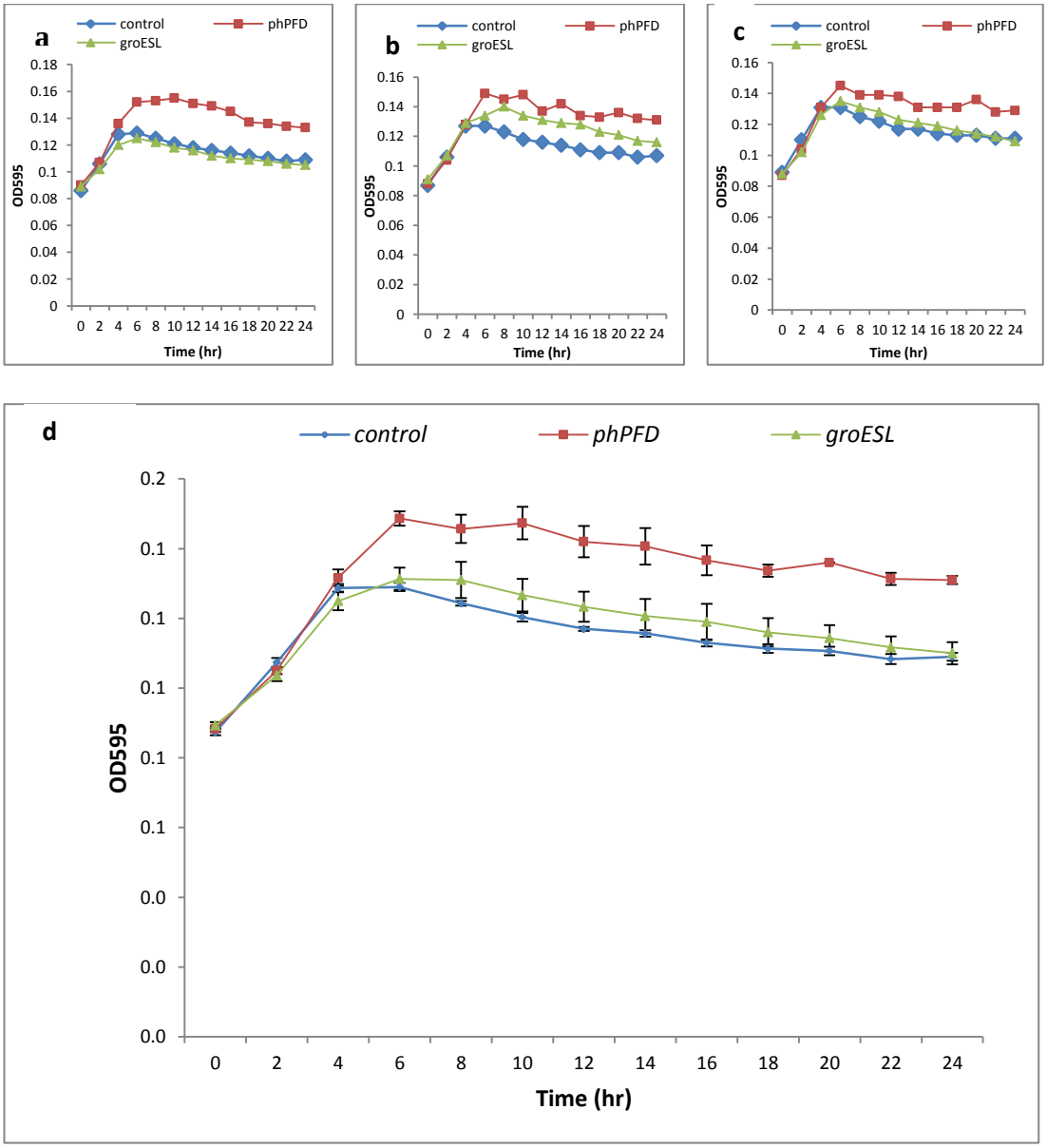


Figure 3.26: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB1C3) in cultures containing 1.1% *n*-butanol.

E. coli MG1655 cells carrying different protein chaperones on a high copy number plasmid were grown in LB containing 1.1% *n*-butanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

At a lower expression level in pSB4K5, both *groESL* and *phPFD* strains increase tolerance to ethanol up to 4% v/v. The growth of the *groESL* strain was nearly identical to the *phPFD* strain (Figures 3.27 – 3.31). When challenged with *n*-butanol, both transformants were more tolerant than the control strain. They both showed a comparable growth at all the concentrations of *n*-butanol tested (Figures 3.32 – 3.36).

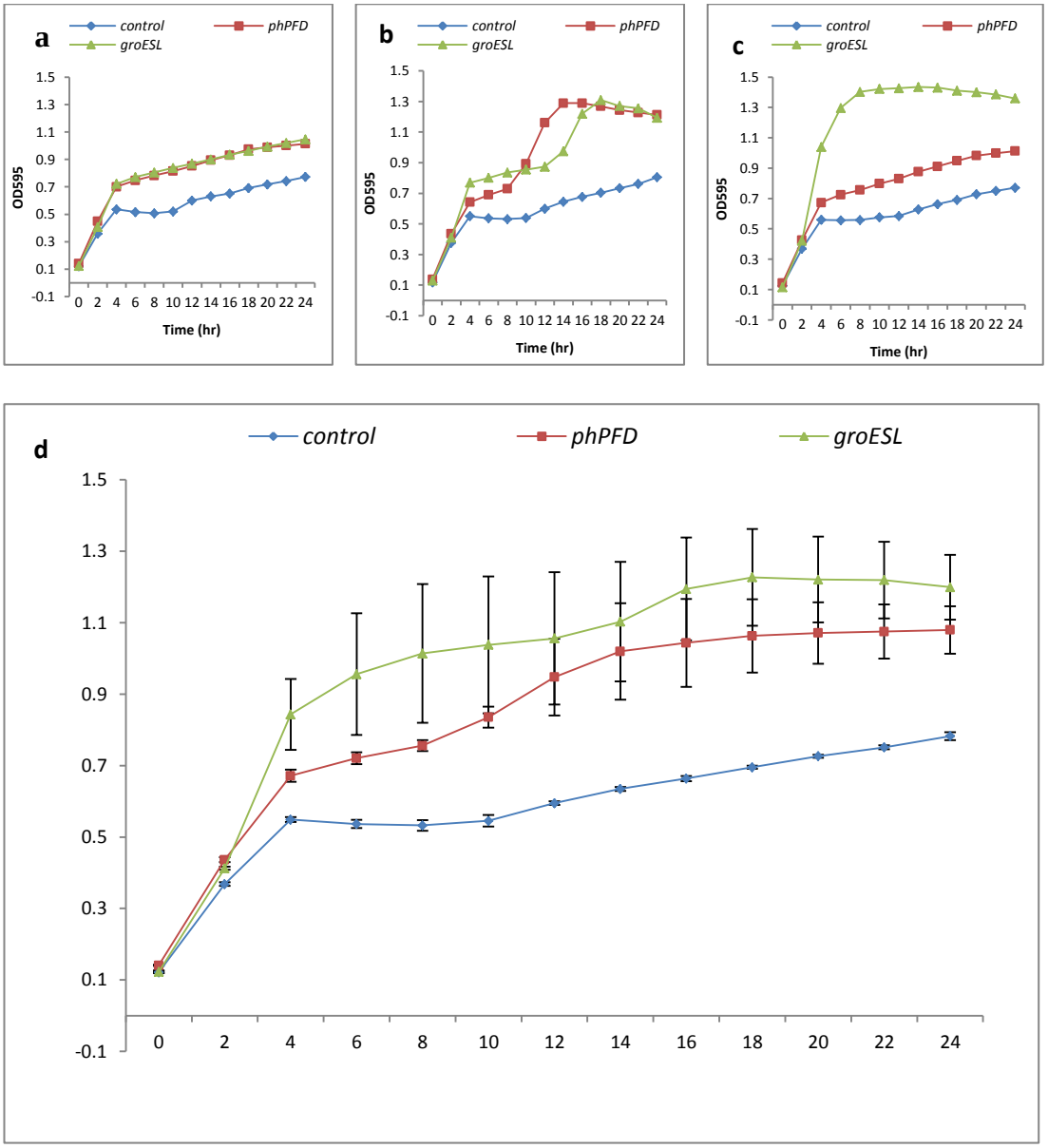


Figure 3.27: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB4K5) in cultures containing 0% ethanol.

E. coli MG1655 cells carrying different protein chaperones on a low copy number plasmid were grown in LB containing no ethanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

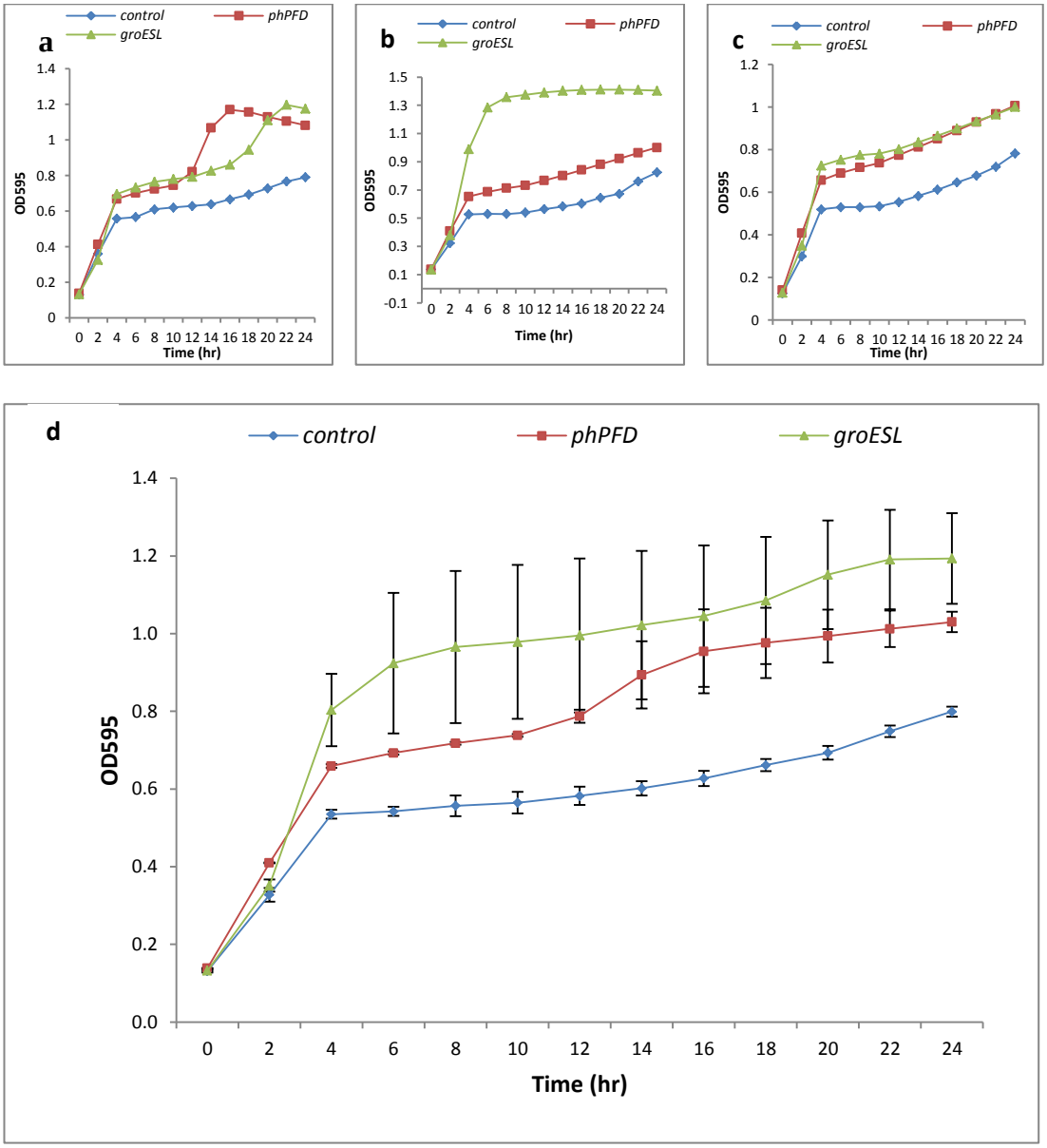


Figure 3.28: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB4K5) in cultures containing 2% ethanol.

E. coli MG1655 cells carrying different protein chaperones on a low copy number plasmid were grown in LB containing 2% ethanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

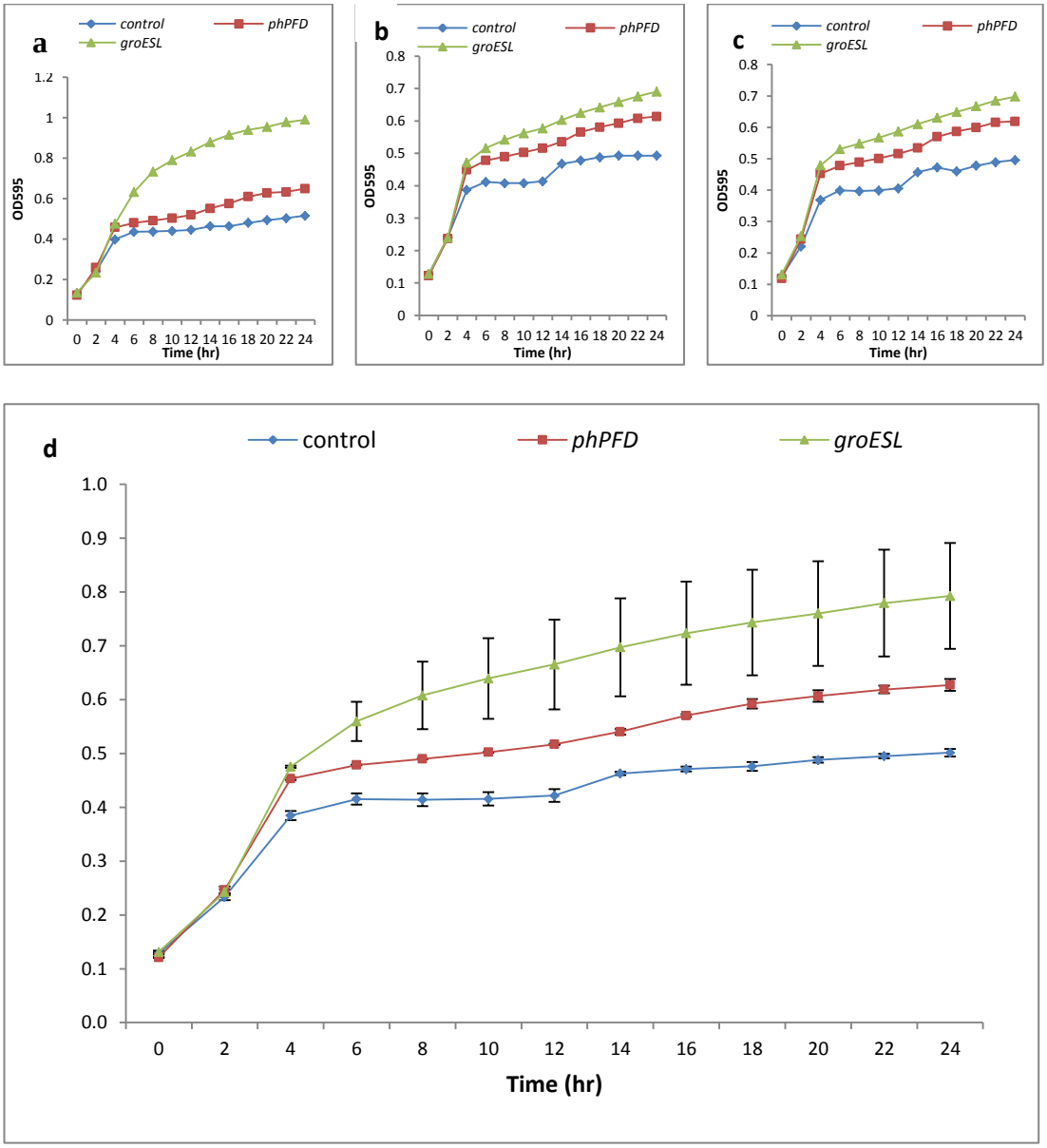


Figure 3.29: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB4K5) in cultures containing 4% ethanol.

E. coli MG1655 cells carrying different protein chaperones on a low copy number plasmid were grown in LB containing 4% ethanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

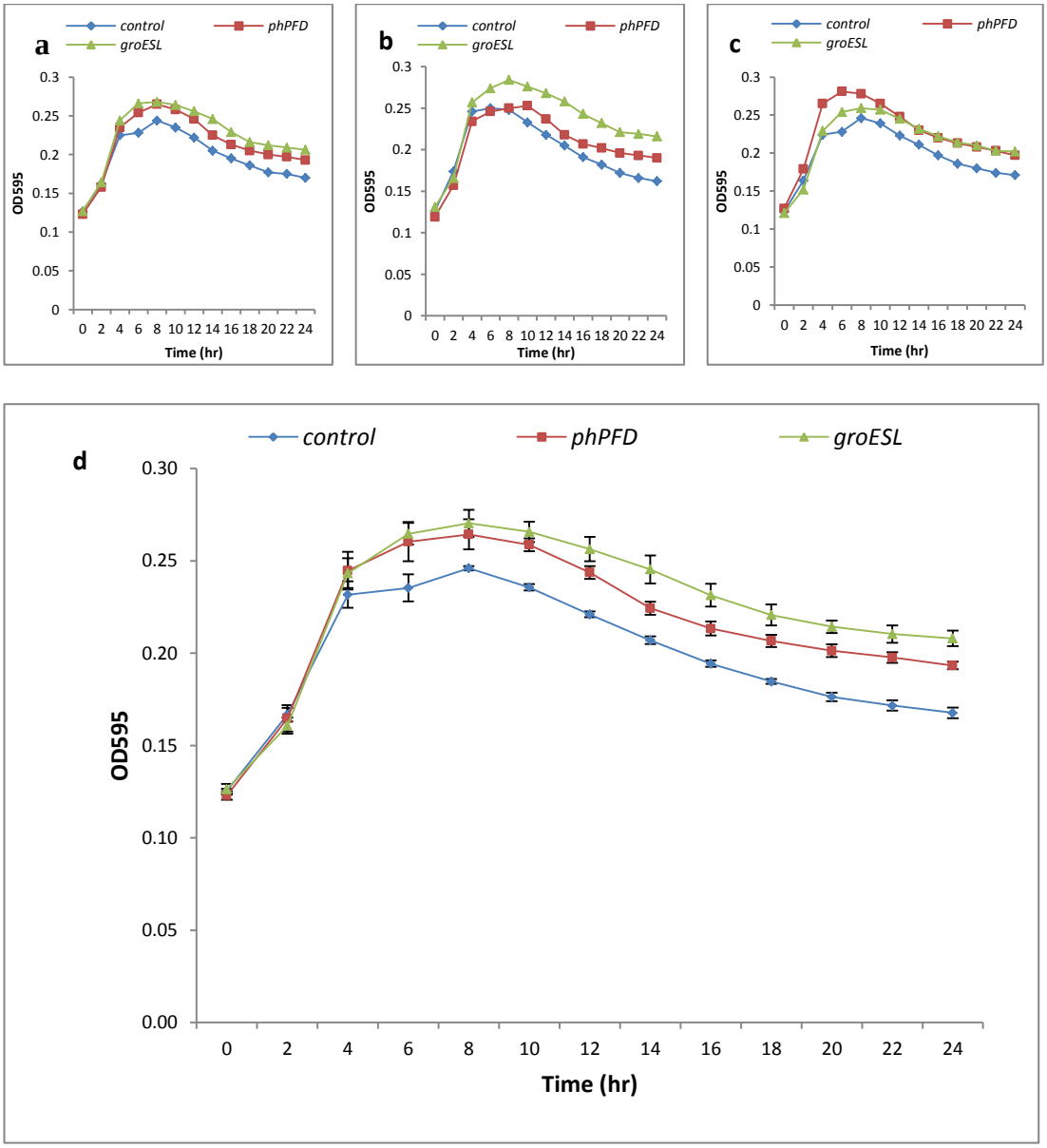


Figure 3.30: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB4K5) in cultures containing 6% ethanol.

E. coli MG1655 cells carrying different protein chaperones on a low copy number plasmid were grown in LB containing 6% ethanol and growth (OD_{595}) was measured at different time points. The *groESL* and *phPFD* strains appear more tolerant to this concentration of ethanol compared to the control strain. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

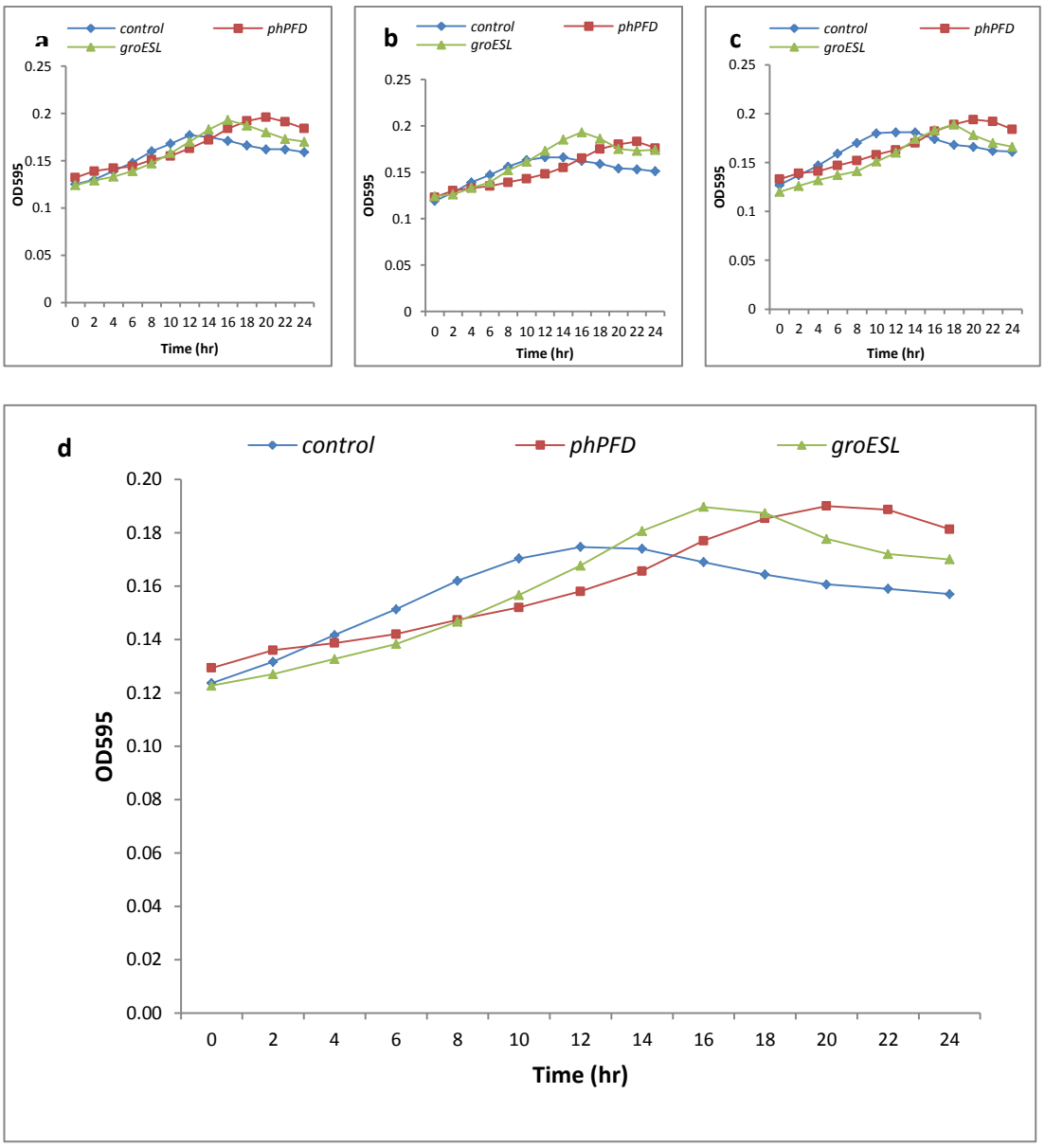


Figure 3.31: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB4K5) in cultures containing 8% ethanol.

E. coli MG1655 cells carrying different protein chaperones on a low copy number plasmid were grown in LB containing 8% ethanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

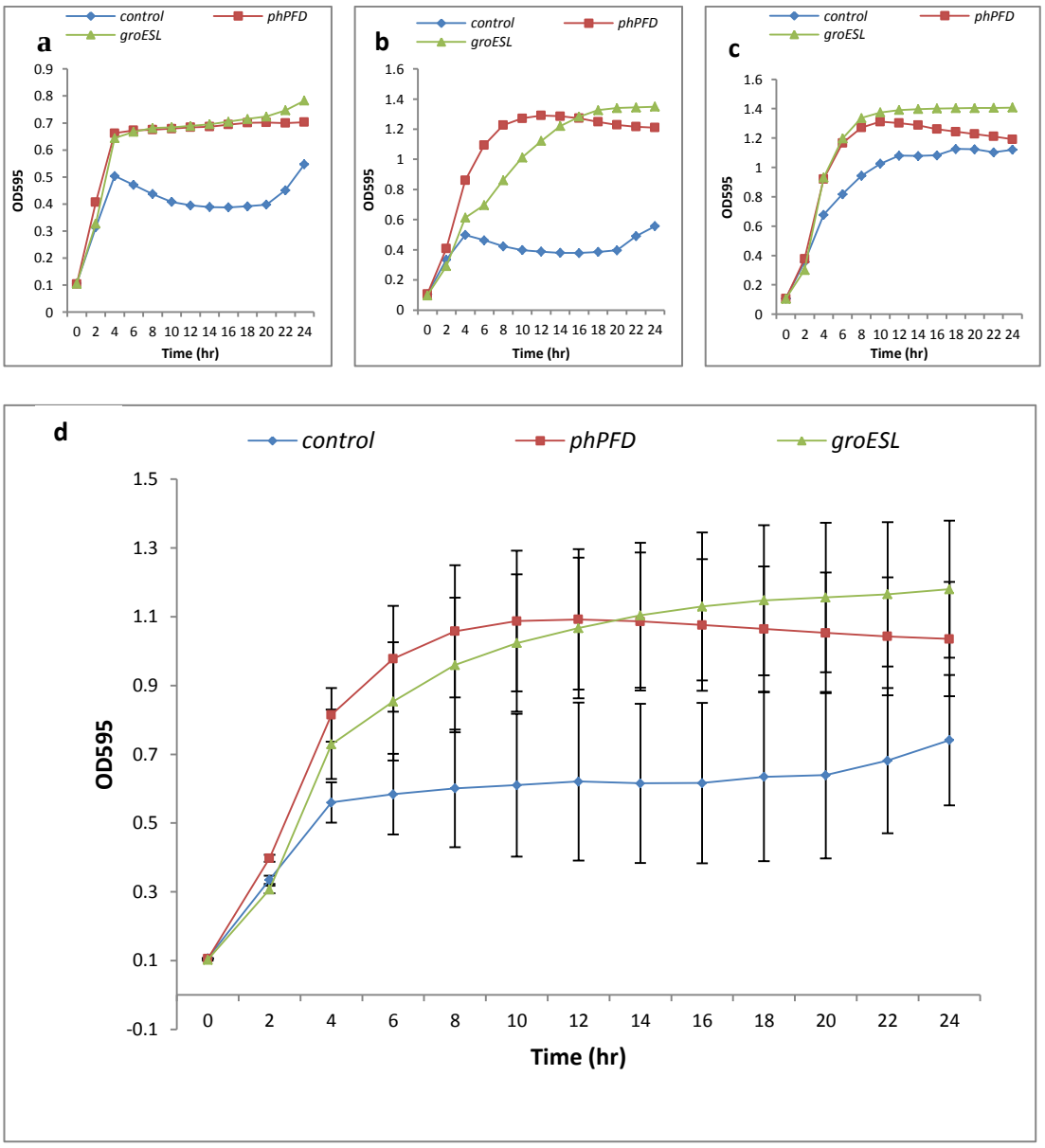


Figure 3.32: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB4K5) in cultures containing 0% *n*-butanol.

E. coli MG1655 cells carrying different protein chaperones on a low copy number plasmid were grown in LB containing no *n*-butanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

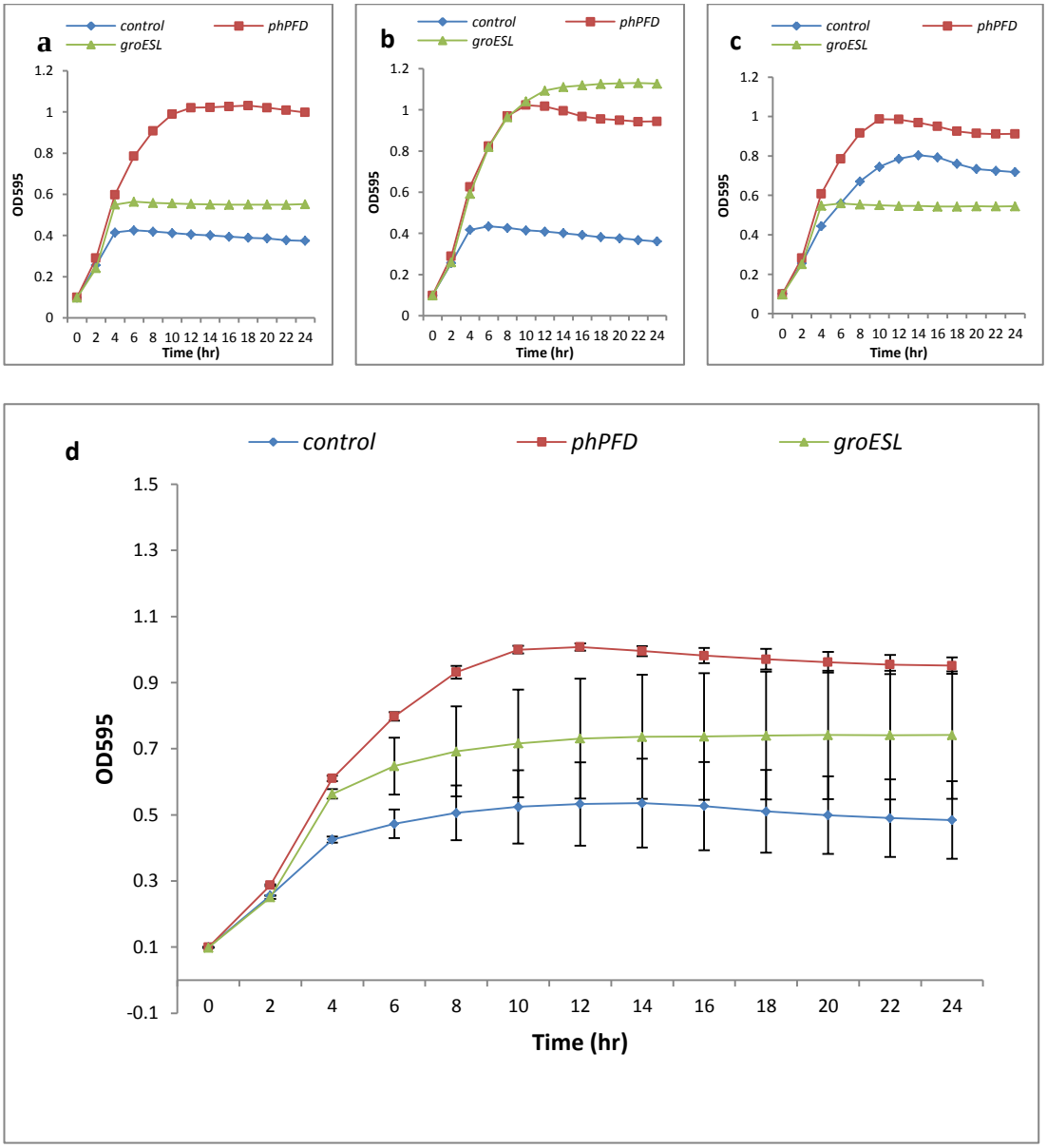


Figure 3.33: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB4K5) in cultures containing 0.5% *n*-butanol.

E. coli MG1655 cells carrying different protein chaperones on a low copy number plasmid were grown in LB containing 0.5% *n*-butanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

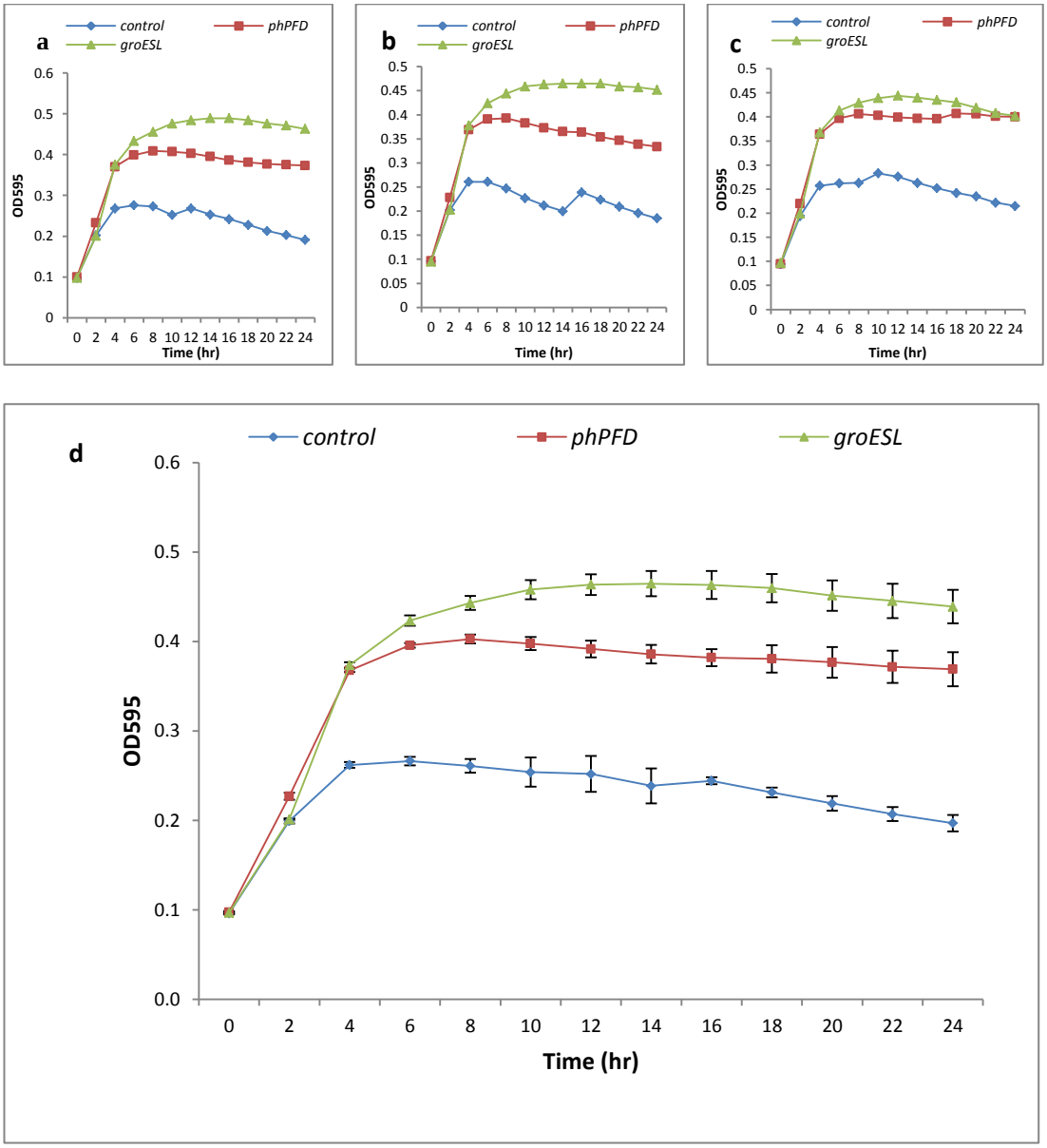


Figure 3.34: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB4K5) in cultures containing 0.7% *n*-butanol.

E. coli MG1655 cells carrying different protein chaperones on a low copy number plasmid were grown in LB containing 0.7% *n*-butanol and growth (OD_{595}) was measured at different time points. The *groESL* and *phPFD* strains show a better growth pattern at this concentration of butanol compared to the control strain. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

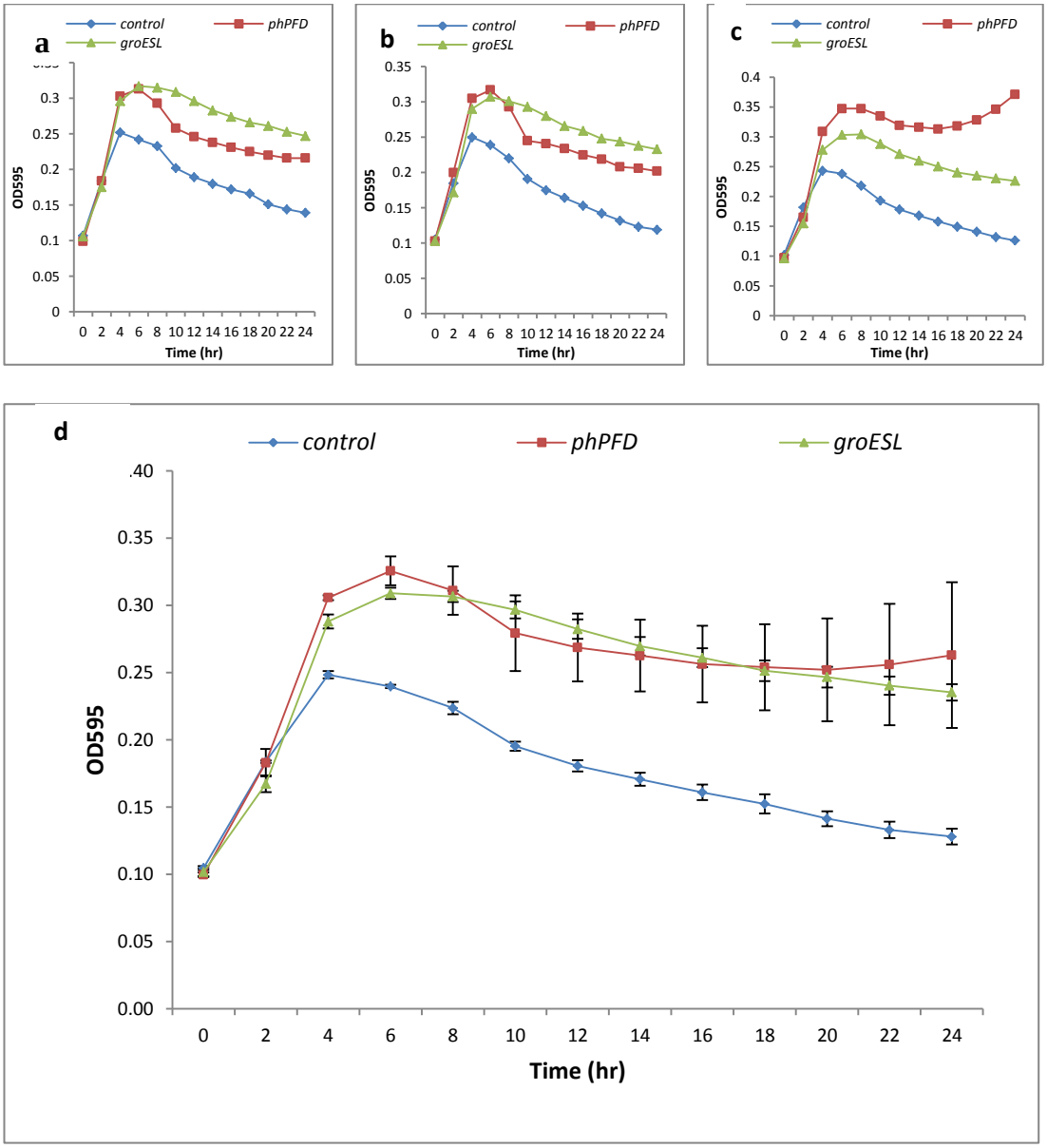


Figure 3.35: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB4K5) in cultures containing 0.9% *n*-butanol.

E. coli MG1655 cells carrying different protein chaperones on a low copy number plasmid were grown in LB containing 0.9% *n*-butanol and growth (OD_{595}) was measured at different time points. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

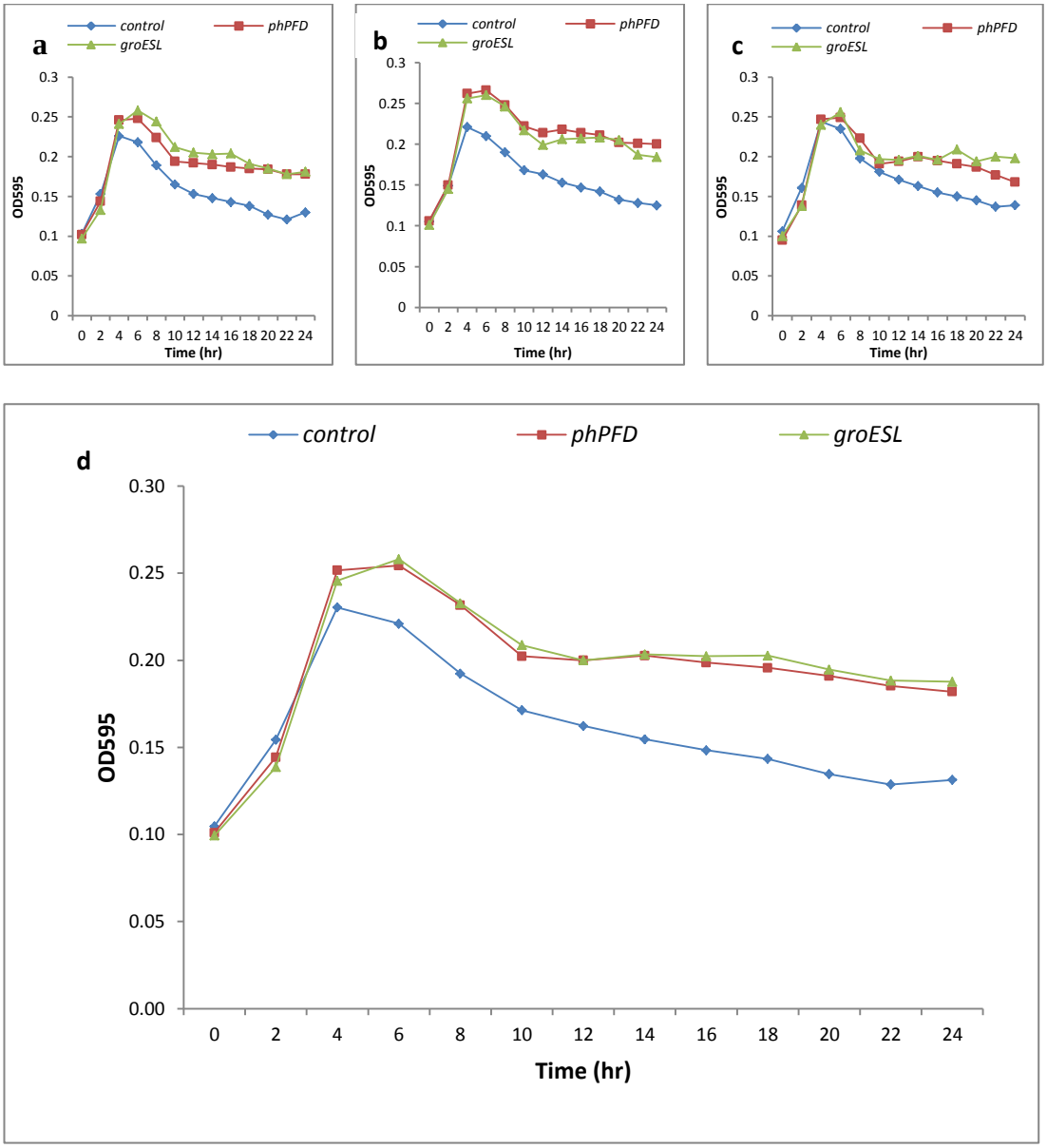


Figure 3.36: Time-course experiments comparing *phPFD* and *groESL* strains (in pSB4K5) in cultures containing 1.1% *n*-butanol.

E. coli MG1655 cells carrying different protein chaperones on a low copy number plasmid were grown in LB containing 1.1% *n*-butanol and growth (OD_{595}) was measured at different time points.Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

3.2.6 Effect of combining two tolerance genes

Since solvent tolerance is a complex phenotype involving several genes, the hypothesis that some of the tolerance genes identified in this work will work in synergy when combined to further increase tolerance to ethanol and *n*-butanol was tested. Several combinations of these genes were tested. As before, the gene combinations were over-expressed in pSB1C3 and induced with the *lac* promoter. A measurement of growth (OD_{600}) and colony forming units (CFU/ml) at 24 hr of incubation in 4% ethanol and 0.5% *n*-butanol were used to determine the percentage survival of the strains harbouring the BioBrick combinations. The average growth was identical for all the strains at 24 hr in the absence of ethanol and *n*-butanol (Figure 3.37). Using the growth assay to determine tolerance, the only combination which appeared to show some additive effect was the *feoA+glpC* combination in the ethanol experiment (Figure 3.37). In Figure 3.37e, this combination was indeed more tolerant to ethanol than the control strain and the strains carrying the individual genes ($p < 0.05$). However, in the viability assay, there was no significant difference in the tolerance of *feoA+glpC* to ethanol compared to the strains overexpressing the individual genes (Figure 3.38d). There were no obvious synergies between all of the other genes combined even though nearly all the combinations tested increased tolerance to ethanol and *n*-butanol. The percentage survival of the combinations was comparable to one or both of the genes individually (Figure 3.38). In some other combinations the percentage survival was lower than that of the individual genes used for the combination as was observed for *marA+phPFD* (Figure 3.38b) and *dps+phPFD* in 0.5% *n*-butanol (Figure 3.38e). *glpC+marA* does not increase tolerance to ethanol even though *glpC* and *marA* individually increase ethanol tolerance ($p < 0.05$) using the colony count assay (Figure 3.38f).

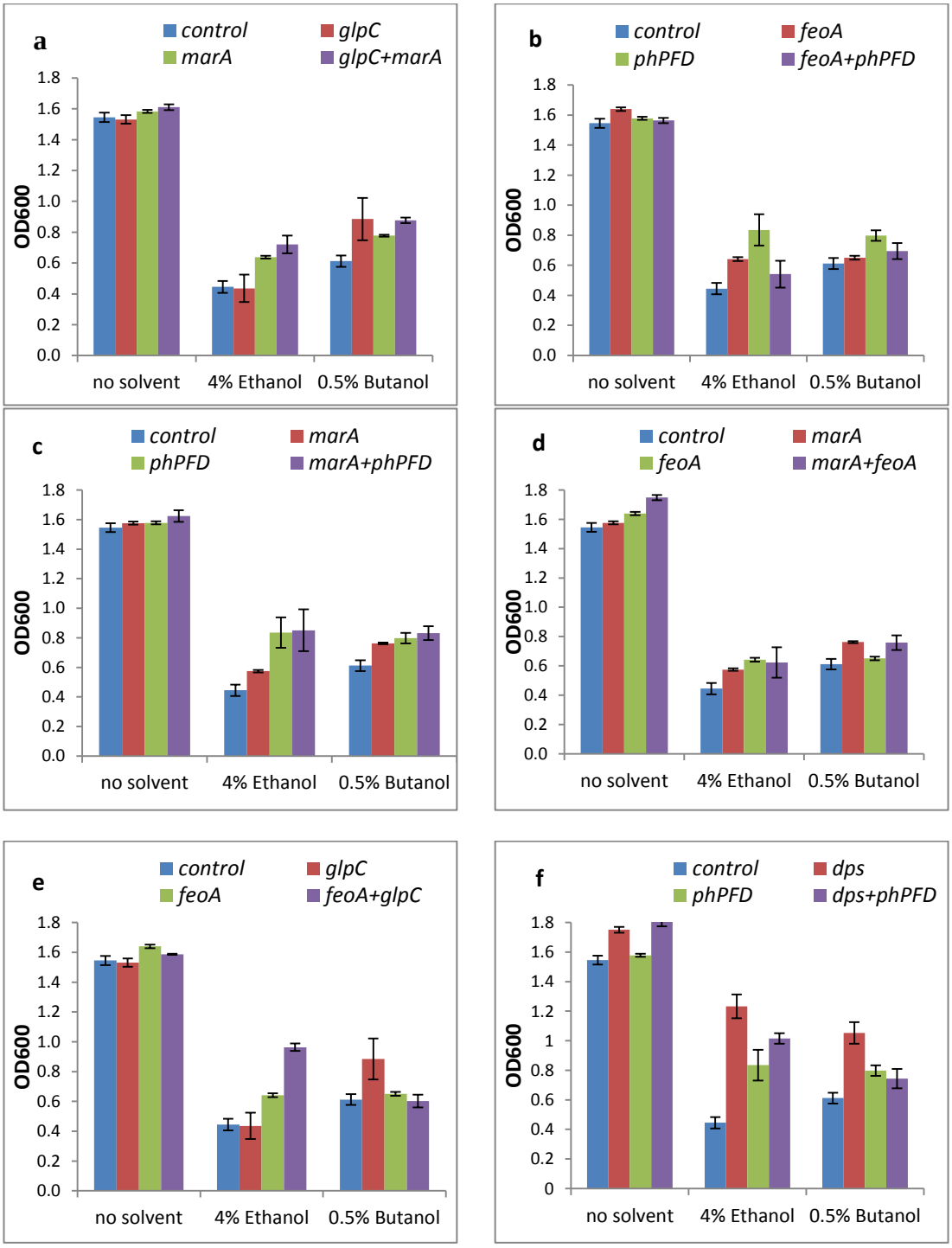


Figure 3.37: Effect of a combination of two stress response genes on solvent tolerance.

Growth of *E. coli* strains carrying a combination of two stress response genes on pSB1C3 was studied under ethanol and *n*-butanol stress after a 24 hr incubation. The experiment was carried out in triplicate. Error bars represent standard error of biological replicates.

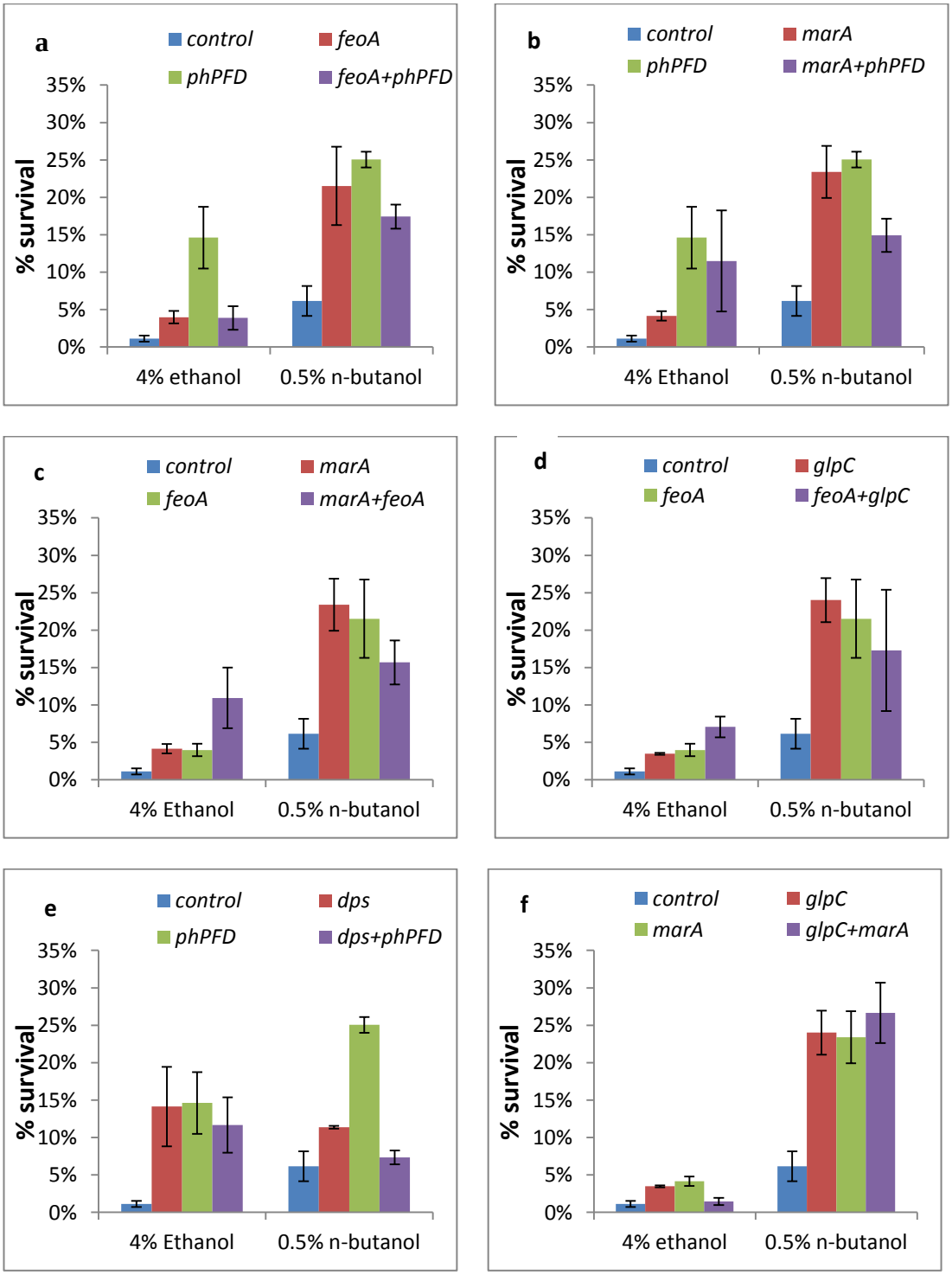


Figure 3.38: Effect of combining two stress tolerance genes.

Viable cell counts (CFU/ml) were performed at 24 hr to determine any possible synergistic interactions between two stress tolerance BioBricks in the presence of ethanol or *n*-butanol. Error bars indicate standard error of three biological replicates, each containing three technical replicates.

3.2.7 Construction of a butanol tolerance device

Failing to observe obvious synergies between the parts combined above, a more rational approach was used to construct a 'butanol tolerance device'. Synergistic interactions that may increase *n*-butanol tolerance was investigated because butanol is a more preferred fuel compared to ethanol since it has a number of properties similar to gasoline, among other advantages. The top four genes that conferred *n*-butanol tolerance in this study and also belonged to different mechanisms of tolerance were chosen and combined into an operon under the control of the *lac* promoter. The chosen genes were *atf1*, *phPFD*, *marA* and *glpC* and were arranged in that order.

In order to reduce the metabolic burden on the cell as a result of overexpressing these four genes, expression of the construct was modulated by using different copy number plasmids and by varying the IPTG concentration used for induction. When IPTG (90 µg/ml) was used to induce the *lac* promoter, cells overexpressing the 'butanol tolerance device' on a low copy number plasmid pSB4K5 attained an optical density (OD_{600}) similar to cells overexpressing the construct on the high copy number plasmid, pSB1C3 (Figure 3.39a). However, Figure 3.39b shows that without the addition of IPTG to the cultures, cells overexpressing the construct on pSB4K5 grew better in *n*-butanol than those carrying the construct on pSB1C3 (p <0.05). Intermediate concentrations of IPTG tested failed to increase tolerance of the butanol tolerance device to *n*-butanol. Therefore, a time-course experiment was carried out to observe growth of the strain carrying a combination of these four genes at different concentrations of *n*-butanol. IPTG was not added to the cultures. The butanol tolerance device did not increase butanol tolerance but displayed growth similar to the control strain at the different concentrations tested (Figure 3.40).

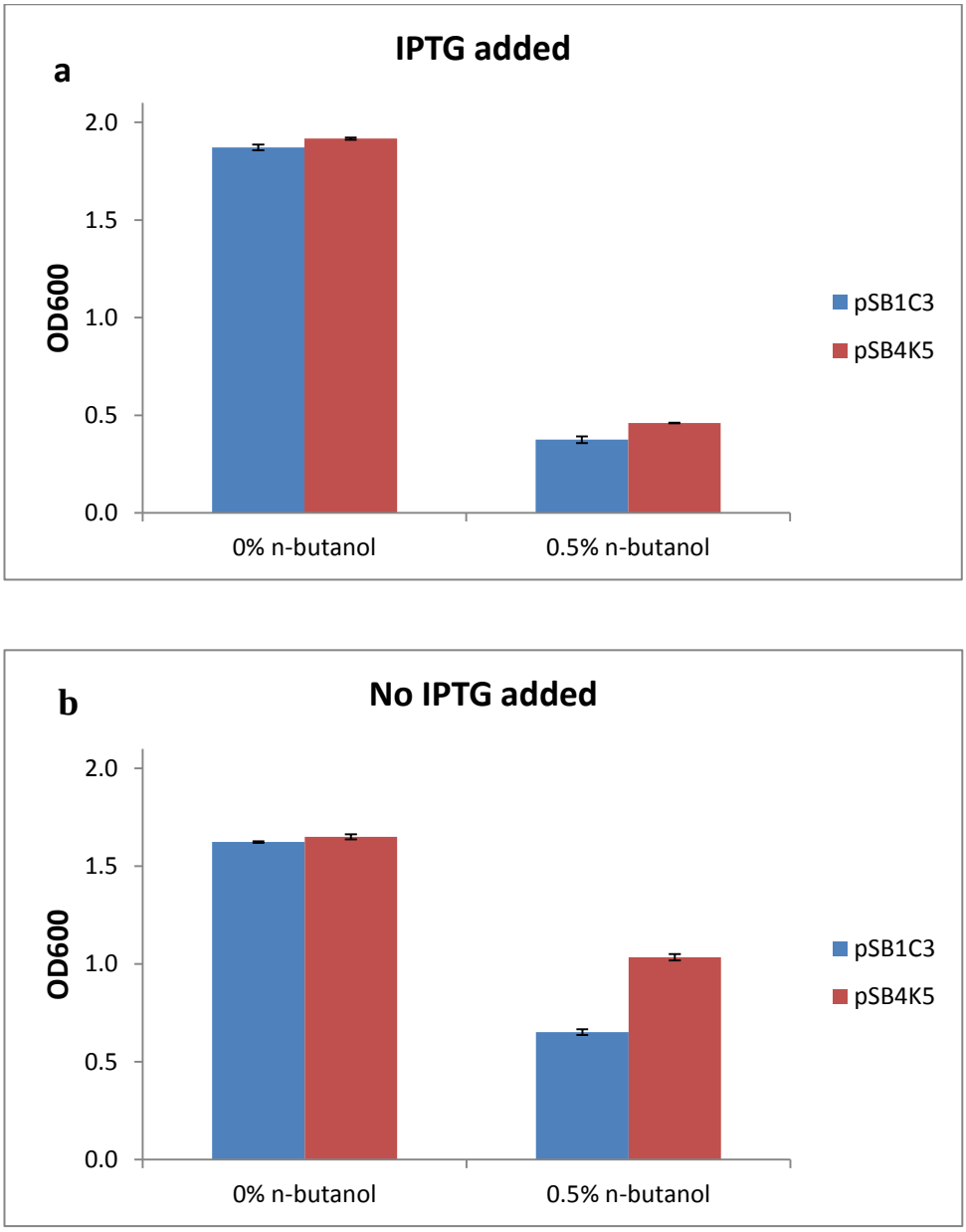


Figure 3.39: Determination of suitable conditions for increasing *n*-butanol tolerance of the butanol tolerance device.

E. coli overexpressing the 'butanol tolerance device' composed of a combination of *atf1*, *phPFD*, *marA* and *glpC* was expressed on pSB1C3 and pSB4K5 in the (a) presence or (b) absence of IPTG induction to determine its effect in increasing tolerance to 0.5% v/v *n*-butanol.

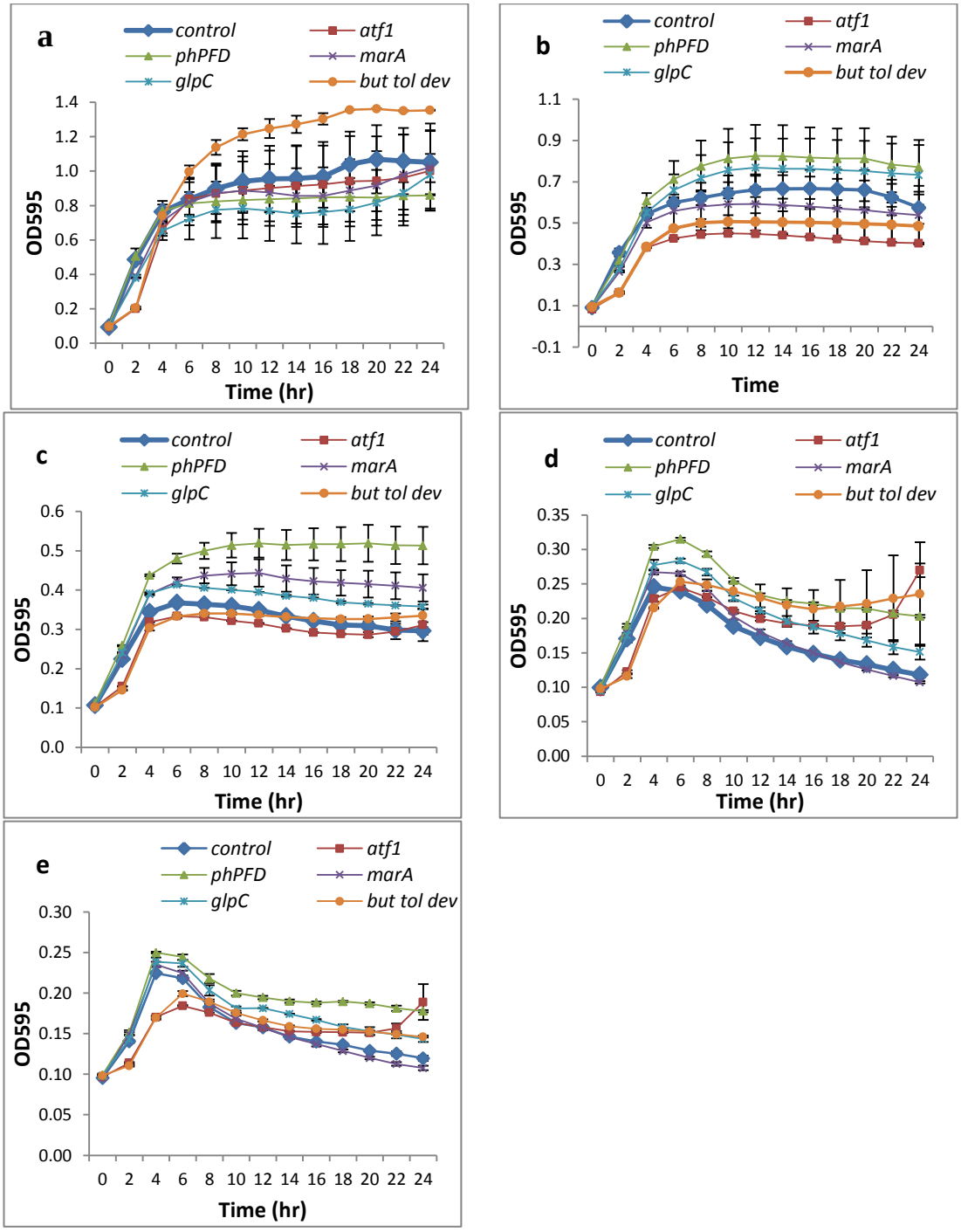


Figure 3.40: Time-course experiments to determine the effect of the butanol tolerance device on *n*-butanol tolerance.

E. coli MG1655 overexpressing a combination of *atf1*, *phPFD*, *marA* and *glpC* were grown in LB containing (a) 0% (b) 0.5% (c) 0.7% (d) 0.9% and (e) 1.1% *n*-butanol. Error bars indicate standard error of three biological replicates.

3.3 Discussion

With recent advances in transcriptional studies and the availability of new molecular tools, it has been possible to identify genes that respond to different stresses. The exact role they play in tolerance mechanisms can only be known by testing them individually. Knowing this will make it possible to develop different tolerance modules for a wide range of organic solvents. For the purposes of this study, a library of potential solvent tolerance genes was generated in the BioBrick format. The library contained genes that play different roles in oxidative stress response, encode membrane-associated proteins and protein chaperones. There were others that encode a DNA-binding transcriptional activator, an exopolysaccharide biosynthesis regulator and an Alcohol acetyltransferase 1. These genes, with the exception of *atf1* and *rcsA* have been shown to respond to stresses induced by presence of some antibiotics (Cohen *et al*., 1989) and organic solvents such as hexane, toluene, cyclohexane and isobutanol (Asako *et al*., 1997; Okochi *et al*., 2007; Okochi *et al*., 2008).

Defining an appropriate assay for measuring tolerance is a key aspect in engineering tolerant strains (Zingaro and Papoutsakis, 2012). Previously, solvent tolerance was assessed by overlaying solid media containing bacterial cells with a solvent being tested and looking out for cell growth (Shimuzu *et al*., 2005). More frequently used methods to assess solvent tolerance are by assaying cell growth (optical density) and by performing colony counts (CFU). These two methods were used in this study to assess ethanol and *n*-butanol tolerance conferred by a library of stress response genes. Using the growth assay, some genes that were shown to increase tolerance to ethanol and to *n*-butanol did not seem to increase tolerance to these alcohols when colony counts were performed. Also other genes such as *glpC* and *sodA* that showed tolerance to ethanol and *n*-butanol respectively in the CFU assay did not show

resistance to these solvents when the cell density assay was used. Discrepancies in the results obtained from these assays are due to the fact that the optical density measures the growth dynamics of a population of cells and the absorbance measurements of these cultures includes light scattered by both live and dead cells so OD_{600} readings do not reflect the actual number of live cells or cells that are resistant to the organic solvents. Colony counts, on the other hand, show the actual number of cells that survive exposure to toxic compounds. The *rcsA* strain, for instance was found to be very tolerant to ethanol when the optical density of the culture was measured. However, the colony count was low and did not show tolerance to ethanol. This particular strain produces exopolysaccharides which was expected to coat the cell membrane and prevent the solvent from damaging the cell. Instead the exopolysaccharides were released into the medium rendering it very turbid and making the strain appear tolerant to ethanol even though it was not. Similarly, there was some variability observed when the plate reader was used to measure growth of cultures which could be attributed to the relative smaller volumes of culture used. Larger volume of cultures in shake flasks should be used in future experiments to confirm some of the plate reader experiments. These differences in results obtained with cell growth and CFU emphasise the need to use alternative approaches to measure tolerance to organic solvents.

Very few studies have used different approaches to measure organic solvent tolerance. In this study, a bioluminescence assay was used as a third method to complement the traditional ways of assessing organic solvent tolerance. This bioluminescence method is rapid and does not require the addition of an aldehyde substrate since the construct used carried the *luxCDE* genes from *Vibrio fisheri.* Several bioluminescence-based assays have been used to detect the presence of toxic

chemicals in water (Gu and Gil, 2001) and to determine the effect of biocides including triclosan on luminescent bacteria (Martin *et al*., 2001; Robinson *et al*., 2011). Bioluminescence determines cell viability by indirectly measuring the cell's metabolic activity in real-time. It was therefore adapted in this study to determine the effect of mild concentrations of toxic compounds on bioluminescent *E. coli* overexpressing different genes implicated in solvent tolerance.

As proof-of-concept that the luminescence assay can be used to screen genes to determine solvent tolerance, the BioBrick library of potential solvent tolerance genes generated for this study was screened against ethanol, *n*-butanol, furfural and acetone by measuring bacterial light production in the presence of these toxic chemicals. Following this screening, the top tolerance genes were identified. These were genes that had consistently increased tolerance to ethanol and *n*-butanol in two or three out of the three methods used to measure solvent tolerance. Ethanol and *n*-butanol tolerance was best achieved by overexpressing the molecular chaperone, Prefoldin, encoded by *phPFD*. This suggests that protein denaturation is a key component of ethanol and *n*-butanol stress and by overexpressing *phPFD*, damage to cellular proteins is reversed resulting in growth even in the presence of these alcohols. Molecular chaperones are produced in cells as a response to heat shock or as a result of protein misfolding or denaturation. To the best of my knowledge, the direct role of Prefoldin in alcohol tolerance has not been reported even though it has been shown that ethanol is a major elicitor of heat shock response (Buttke and Ingram, 1984; Camarena *et al*., 2010). Heat shock response has been shown to be highly beneficial in ethanol tolerance (Goodarzi *et al*., 2010) confirming an overlap in heat shock and solvent stress responses. Prefoldin is a hexameric chaperone originally isolated from the thermophile *Pyrococcus horikoshii* OT3, and like other molecular

chaperones, it is known to increase tolerance to high temperatures by refolding denatured proteins showing a potential role in increasing resistance to heat shock. The role of Prefoldin in solvent tolerance was first reported by Okochi *et al*. (2008) who demonstrated that *E. coli* tolerance to hexane could be increased by overexpressing *phPFD*. Prefoldin from *Pyrococcus horikoshii* OT3 binds and delivers unfolded proteins not only to group II chaperonins found in archaea but also to group I chaperonins to correct protein folding (Okochi *et al*., 2002) Both chaperone and chaperonin are required for correct folding. However, overexpressing Prefoldin alone increased tolerance to ethanol and *n*-butanol. This implies that Prefoldin may recruit a native *E. coli* group I chaperonin.

A native *E. coli* chaperone, *groESL* was unable to increase tolerance to ethanol and *n*-butanol when highly expressed. When its overexpression was limited by placing it in a low copy number vector, it was able to increase tolerance to ethanol similar to *phPFD*. It was also tolerant to *n*-butanol comparable to *phPFD* suggesting that the beneficial effect of *groESL* is only observed at a low expression. It is likely that the mechanism by which *groESL* protects solvents from *n*-butanol stress is similar to that used by *phPFD*. It will still be interesting to investigate if these chaperones have specific protein substrates and if they do, it will be important to identify such substrates which can be protected from ethanol and *n*-butanol stress during design and engineering of tolerant strains. Similarly, Zingaro and Papoutsakis (2013) showed that *groESL* when overexpressed on a low copy number plasmid increased tolerance to several alcohols including ethanol and *n*-butanol. However, in that report contrary to this study, ethanol and *n*-butanol tolerance was not achieved when *groESL* was placed under the control of the *lac* promoter. The authors of that work

managed to increase tolerance to these alcohols only when expression was driven by the native *groESL* promoter.

Strains over-expressing *manXYZ* and *sodA* were more tolerant to ethanol than the control strain. *manXYZ* has been previously shown to be upregulated in ethanol tolerant strains of *E. coli* (Horinouchi *et al.*, 2010) suggesting a link between this operon and ethanol resistance. The *manXYZ* operon encodes a mannose transporter localised in the cell membrane and might increase tolerance to ethanol by modifying the membrane properties of the cell (Okochi *et al.*, 2007). Relatively high survival of the *sodA* strains suggests that oxidative stress protection may play a role in ethanol tolerance. Surprisingly, *n*-butanol tolerance as a result of oxidative stress response was not observed in this study implying that the effect of butanol on the cell differs from the effect of ethanol in some ways. Consistent with a different study, *sodA* and *manX* are downregulated during butanol stress while *marA* is highly upregulated (Zhang *et al.*, 2012). Overexpressing *marA* and a gene encoding a membrane-associated protein such as *glpC* increased *n*-butanol tolerance. *glpC* has been previously shown to increase tolerance to other organic solvents such as *n*-hexane, cyclohexane and toluene (Shimuzu *et al.*, 2005) possibly by modifying the membrane properties to limit entry of the solvent into the cell. The *glpC* gene, one of the genes belonging to the *glpABC* regulon, encodes an anaerobic glycerol-3-phosphate dehydrogenase (Shimuzu *et al.*, 2005). Being non-catalytic, it resides in the cell envelope and anchors the catalytic dimer, *glpAB* (Cole *et al.*, 1988) and might be essential in protecting the cell membrane from damage (Shimuzu *et al.*, 2005) possibly by assuming a conformation that alters its interaction with the phospholipids in the inner membrane and in effect reducing the membrane fluidity. This suggests that membrane damage is prominent during *n*-butanol stress, thus,

protecting the cell's membrane is important in rationally engineering solvent tolerant microbial hosts. *feoA*, encoding a ferrous iron transporter, was found to increase ethanol tolerance even though its effect on *n*-butanol tolerance was not noticeable contrary to an earlier report by Reyes *et al*., (2011) implicating *feoA* in *n*-butanol tolerance. FeoA has not been previously associated with ethanol tolerance. The role of ferrous iron metabolism in alcohol tolerance remains unclear. However, *feoA* has been proposed to interact with the negatively charged cytoplasmic membrane due to the high pI of the former (Cartron *et al*., 2006). This will therefore imply that *feoA* can also potentially modify the membrane properties of the cytoplasmic membrane to increase alcohol tolerance. Overexpression of *marA* was shown to increase tolerance to ethanol stress when the cell growth and CFU assays were used but when the luminescence assay was used to determine tolerance, it did not show an increase to ethanol tolerance. The time-course experiments showed that *marA* had a long lag phase when challenged with ethanol and *n*-butanol (Figures 3.10 and 3.11). Therefore, *marA*-overexpressing strains will appear less tolerant to these alcohols when tolerance is assayed during this phase of growth and this explains why ethanol tolerance was not observed when the luminescence assay was used since the luminescence assay was used to measure tolerance 5 hr after ethanol was added to the culture. Chong *et al*., (2013) has also reported that *marA* is one of the genes required by an ethanol tolerant *E. coli* strain to resist ethanol stress. Tolerance of *marA*-overexpressing strains to *n*-butanol was observed in all three assays used. Constitutive expression of *marA* resulted in increased expression of *acrAB* and *tolC* (Barbosa and Levy, 2000) which has been linked to efflux and a consequential tolerance to antibiotics such as fluoroquinolones, tetracycline and chloramphenicol

(Cohen *et al*., 1989). This implies that the resistance of *marA* strains to ethanol and *n*-butanol might be due to the extrusion of these alcohols out of the cell.

The involvement of *gorA* in solvent tolerance has not been previously investigated. The sensitivity of *gorA*-overexpressing strains to *n*-butanol as shown in this work could be explained by the fact that since *gorA* inactivates the hydrogen peroxide stress response regulator (OxyR) by reducing the cysteine groups on the regulator (Aslund *et al*., 1999), overexpressing it might prevent OxyR from inducing the transcription of beneficial genes. This observation might also confirm an overlap between *n*-butanol stress response and hydrogen peroxide stress response.

Furfurals have been proposed to inhibit *E. coli* growth by depleting intracellular levels of NADPH (Miller *et al*., 2009) and have been shown to inhibit key glycolytic enzymes such as hexokinase in yeast (Banajeree *et al*., 1981). It was therefore not surprising that none of the constructs in the BioBrick library protected the cells from furfural toxicity. This implies that furfural does not have any grave effect on any of the major cellular components such as the membranes, DNA and proteins. A possible way of eliminating the toxic effects of furfural will be to engineer pathways that will degrade furfural into a less toxic compound (Liu *et al*., 2008; Liu and Moon, 2009). It has also been proposed that over-expressing efflux pumps might be able to exclude inhibitors such as furfurals from the cell and in effect increase tolerance (Dunlop *et al*., 2011). None of the constructs tested was able to increase tolerance to acetone suggesting that acetone might be inhibiting growth using other mechanisms. This re-emphasises the need to tailor different tolerance module to different organic solvents.

The possibility of finding synergistic or additive interactions between the tolerance genes was also explored. Despite several attempts to vary expression levels of these

gene combinations, no obvious synergies were observed even though most of the combinations increased tolerance to ethanol and to *n*-butanol. Further investigation is required and this challenge highlights the complexity of the organic solvent tolerance phenotype.

In this chapter, using multiple assays including a bioluminescence assays, a library of potential solvent tolerance genes which had not been previously been associated with ethanol, *n*-butanol, furfural and acetone tolerance were screened. By screening this library, further insight into possible mechanisms of toxicity of these inhibitors has been gained. Proof-of-concept tests show that damage to protein and DNA possibly by oxidative stress occurs during ethanol stress and *n*-butanol toxicity is mainly due to membrane and protein damage. The bioluminescence assay can be further developed to screen a wider array of genes against different inhibitory substances in a high throughput fashion. The inability to obtain a butanol tolerance module by combining multiple tolerance genes shows how complicated the effect of organic solvents on the cell is. In-depth knowledge of the precise effects of these organic solvents on bacteria is required to practically engineer tolerant hosts. The effect of *n*-butanol on the cell envelope of *E. coli* is looked at in the next chapter.

Chapter 4: Characterisation of the effects of *n*-butanol on the cell envelope of *E. coli*

4.1 Introduction

The cell membrane of bacteria acts as a potent barrier that regulates the movement of solutes and other compounds into and out of the cell. It is also the first point of contact of stresses from the environment including changes in pH, temperature, osmolarity and solvent toxicity. Organic solvent toxicity in microorganisms has gained a lot of attention in current research since this is a crucial factor that limits productivity of microbial hosts used for various bioprocesses including the production of biofuels and other industrially important chemicals. Some membrane-associated genes upregulated during solvent stress have been shown to increase tolerance to organic solvents (previous chapter) when overexpressed in microbial hosts, re-emphasising the fact that the membrane is targeted by organic solvents.

The effect of ethanol on the cell membrane has been extensively studied due to the economic importance of the alcohol. Ethanol interacts with the cell membrane, resulting in increased membrane fluidity and impaired membrane functions (Cartwright *et al*., 1986; Huffer *et al*., 2011; Sikkema *et al*., 1995). In yeasts it has been shown that key proteins involved in uptake of sugars are inactivated by ethanol (Carlsen *et al*., 1991). Butanol, a biofuel superior to ethanol, in many ways, is more toxic than ethanol due to its longer carbon chain. However, very little is known about its effect on the cell membrane and how it is able to cross the membrane into the cell. Understanding the effect of butanol on the cell envelope is crucial for designing novel strategies to counteract its toxicity.

Gram negative organisms such as *E. coli*, unlike gram positive bacteria, have an outer membrane and a thin peptidoglycan layer in the periplasm in between the outer and inner membranes. The outer membrane is asymmetrical and composed of phospholipids in the inner leaflet of its bilayer whereas the outer leaflet is made up mainly of lipopolysaccharides (LPS) which create a hydrophilic environment limiting the transport of compounds into the cell depending on how hydrophobic they are. The structure of the LPS has been well-studied and characterised (Strain *et al*., 1983). It is made up of three components – the lipid A which anchors the LPS into the hydrophobic region of the outer membrane, a core oligosaccharide called 2-keto-3-deoxyoctonic acid (KDO) and an O-antigen attached to the oligosaccharide which extends into the cell's environment (Raetz and Whitfield, 2002). Newly synthesised LPS molecules are transported to the outer membrane in a process chiefly mediated by the LptA protein (Chng et al., 2010). The core oligosaccharide is negatively charged and in order to prevent repulsion between adjacent LPS molecules, Mg^{2+} bridges adjacent LPS molecules and stabilises the membrane. Anything that interferes with the interaction destabilises the membrane and results in membrane damage. Metal chelators such as EDTA have been shown to release LPS molecules from the outer membrane by chelating the Mg^{2+} ions that maintain the LPS-LPS interaction (Alakomi *et al*., 2006).

Several studies have shown that bacteria respond to membrane stress by modifying the membrane, mainly by altering the structure and composition of its fatty acids. Little is known about the effect of alcohols on other structures of the membrane and how they are able to break this barrier to enter the cell.

The objective of this study is to investigate the exact effect of *n*-butanol on the cell membrane and propose a possible mechanism explaining how *n*-butanol crosses the cell envelope into the cytoplasm. The possibility of increasing butanol tolerance by supplementing the media with divalent metal ions is also covered in this chapter. In order to study this in great detail, enzyme assays were used to detect membrane damage and the purpald assay was employed to detect LPS removal. A novel bioreporting system was developed to further characterise outer membrane damage. Results from these experiments will provide new insights into new strategies that can be used to strengthen the cell membrane to prevent or reduce the toxic effects of organic solvents such as *n*-butanol.

4.2 Results

4.2.1 Detection of outer membrane damage

The alkaline phosphatase assay was performed to determine the effect of *n*-butanol on the outer membrane of *E. coli*. When the outer membrane is damaged, periplasmic contents leak out into the liquid media. By testing for the presence of components of the periplasm in the supernatant of the liquid culture, it will be possible to determine the extent of damage done to the membrane by *n*-butanol. In this experiment, leakage of a periplasmic enzyme, alkaline phosphatase, was determined. Initially, it was very difficult to detect the presence of this enzyme using cells in the exponential phase of growth since the enzyme is not expressed at this phase and levels detected were almost negligible. Work done by Heppel *et al*., (1961) and Hong *et al*., (2007) shows that the *phoA* gene which encodes alkaline

phosphatase in *E. coli* is expressed when cells are starved of phosphate or during the stationary phase of cells when most of the phosphate in the media has been used up. To get around this problem, the *phoA* gene was cloned from *E. coli MG1655*, placed downstream of the *lac* promoter+*lacZ* α′ gene and overexpressed on pSB1C3.

Cultures were grown to the exponential phase and exposed to 1% *n*-butanol, 8 mM EDTA, 8 mM SDS or water ('no solvent' control) for 1 hr and the supernatants of these cultures were assayed for the presence of alkaline phosphatase. A high enzyme activity in the supernatant will signify membrane leakage allowing the free enzyme to react with and convert the pNPP substrate into product. The effect of EDTA and SDS were included because these have been shown to damage cell membranes (Hardaway and Buller, 1979; Woldringh and van Iterson, 1972) and act as ideal controls. EDTA targets the outer membrane whereas SDS dissolves both inner and outer membranes.

Figure 4.1 shows that when *E. coli* cells were exposed to *n*-butanol for 1 hr, there was some alkaline phosphatase activity in the supernatant which was significantly higher than that ofthe 'no solvent' control (p = 0.025). However, the alkaline phosphatase activity was much lower compared to the culturestreated with SDS (Figure 4.1a). It would have been expected that cells treated with EDTA would have a high enzyme activity in the supernatant since EDTA is a known outer membrane damaging agent but those cultures had a very low enzyme activity (p = 0.128) even though excess (4 times as much EDTA) of Mg^{2+} was added to the supernatants used for the assay to prevent EDTA from inhibiting the alkaline phosphatase.

In a separate experiment conducted at the same time, the permeability of the cell membrane induced by *n*-butanol was investigated by assaying whole cells treated

with *n*-butanol for alkaline phosphatase activity. The rationale behind using whole cells was to check if *n*-butanol rather than causing the enzyme to leak out, made small 'holes' in the membrane as it traversed it into the cytosol via the periplasm. This way, the pNPP substrate will be able to penetrate the membrane and react with the enzyme to form a product which can be measured. As shown in Figure 4.1b the alkaline phosphatase activity of whole cells exposed to *n*-butanol, EDTA and SDS were similar to the 'no solvent' control. Using whole cells for this enzyme assay to determine membrane damage was not the best approach since the substrate is capable of crossing the membrane through porins in the membrane (Martinez *et al*., 1996). Therefore, assuming *n*-butanol made 'holes' in the membrane, the amount of product formed in cells exposed to *n*-butanol will be identical to that formed in the cells with intact membranes.

It was necessary to determine the effect of *n*-butanol on the alkaline phosphatase activity as this could affect the interpretation of the results. To do this, a crude extract of alkaline phosphatase was treated with increasing concentrations of *n*-butanol for 1 hr and the enzyme activity was determined as before. Figure 4.2 shows that the *n*-butanol used in this experiment did not inhibit the alkaline phosphatase activity.

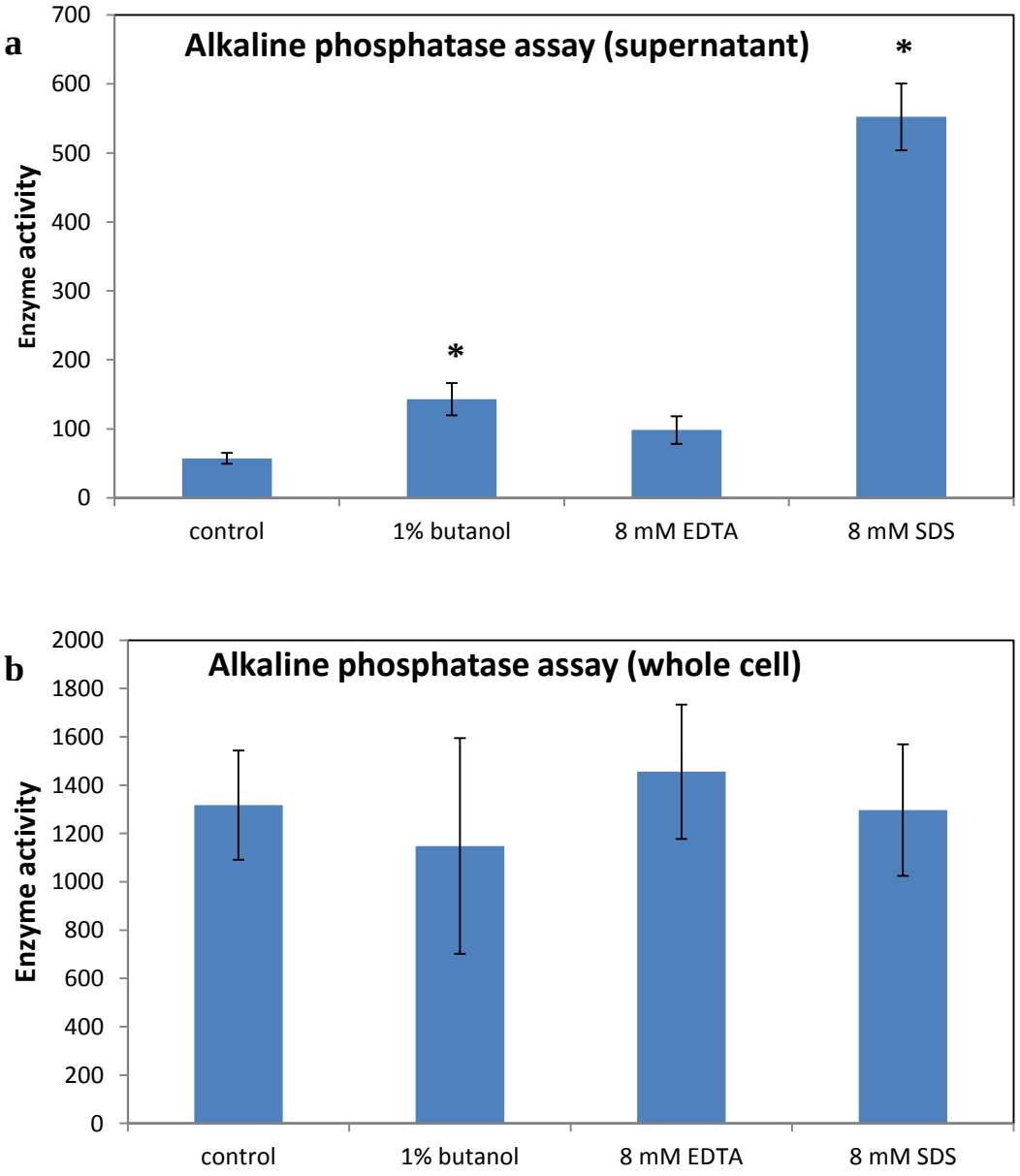


Figure 4.1: Effect of *n*-butanol on the outer membrane of *E. coli*.

Alkaline phosphatase activity was measured using a) culture supernatants and b) whole cells. Control experiments were carried out with EDTA and SDS. Experiments were done in triplicate. Standard errors are shown with the error bars. Asterisk signifies statistical significance ($p < 0.05$).

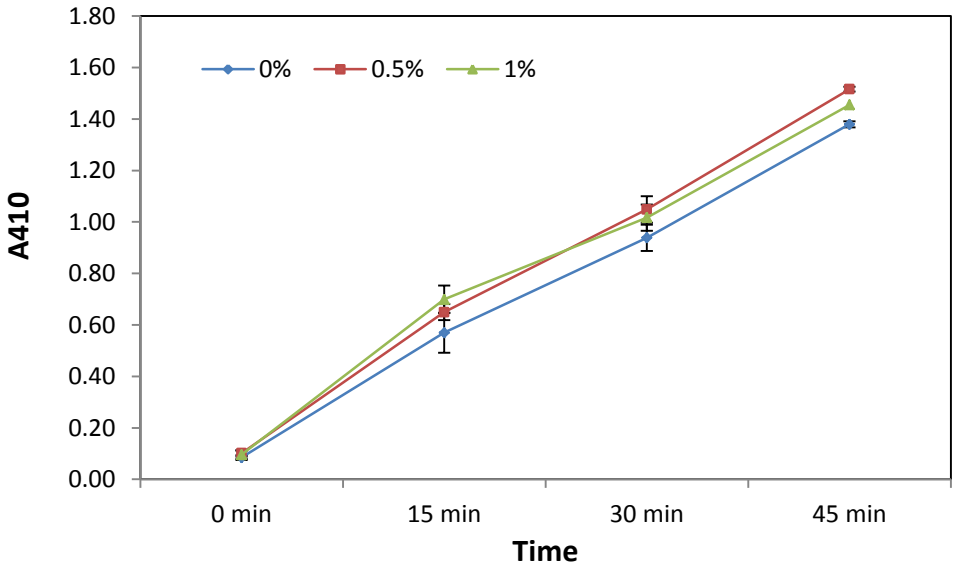


Figure 4.2: Effect of *n*-butanol on alkaline phosphatase activity.

Alkaline phosphatase activity was assayed in different concentrations of *n*-butanol (0%, 0.5% and 1%) to determine enzyme inhibition. Absorbance was measured at 410 nm every 15 min for 45 min. Experiments were done in triplicate. Standard errors are shown with the error bars.

4.2.2 Detection of inner membrane damage

The effect of *n*-butanol on the inner membrane was also investigated. In this experiment the release of a cytosolic enzyme, β-galactosidase, into the liquid culture was determined. Cells were exposed to *n*-butanol, EDTA, SDS or water (control) as described above. Using the Miller assay, the effect of *n*-butanol on the inner membrane was studied. β-galactosidase activity was detected in the supernatant of the cultures exposed to *n*-butanol (Figure 4.3a).

The activity was very low and was not significantly higher (p = 0.170) than the control cells that were not exposed to any of these chemicals. Cells treated with EDTA had an enzyme activity quite identical to the control. This was expected since EDTA has no effect on the inner membrane. Cells treated with SDS had a very high enzyme activity (p = 0.001) as expected. Since the β-galactosidase activity of cells exposed to *n*-butanol was very low, it was assumed that the exposure time might have been too short so the experiment was repeated but this time the cells were exposed to these chemicals for 2 hr instead of 1 hr (Figure 4.4). Though low, supernatant of cultures treated with *n*-butanol had an enzyme activity significantly higher than that of the 'no solvent' control cultures (p = 0.002) suggesting that the effect of the solvent on the inner membrane is not immediate.

The enzyme activity of whole cells exposed to *n*-butanol, EDTA and SDS were similar to the 'no solvent' control (Figure 4.3b).

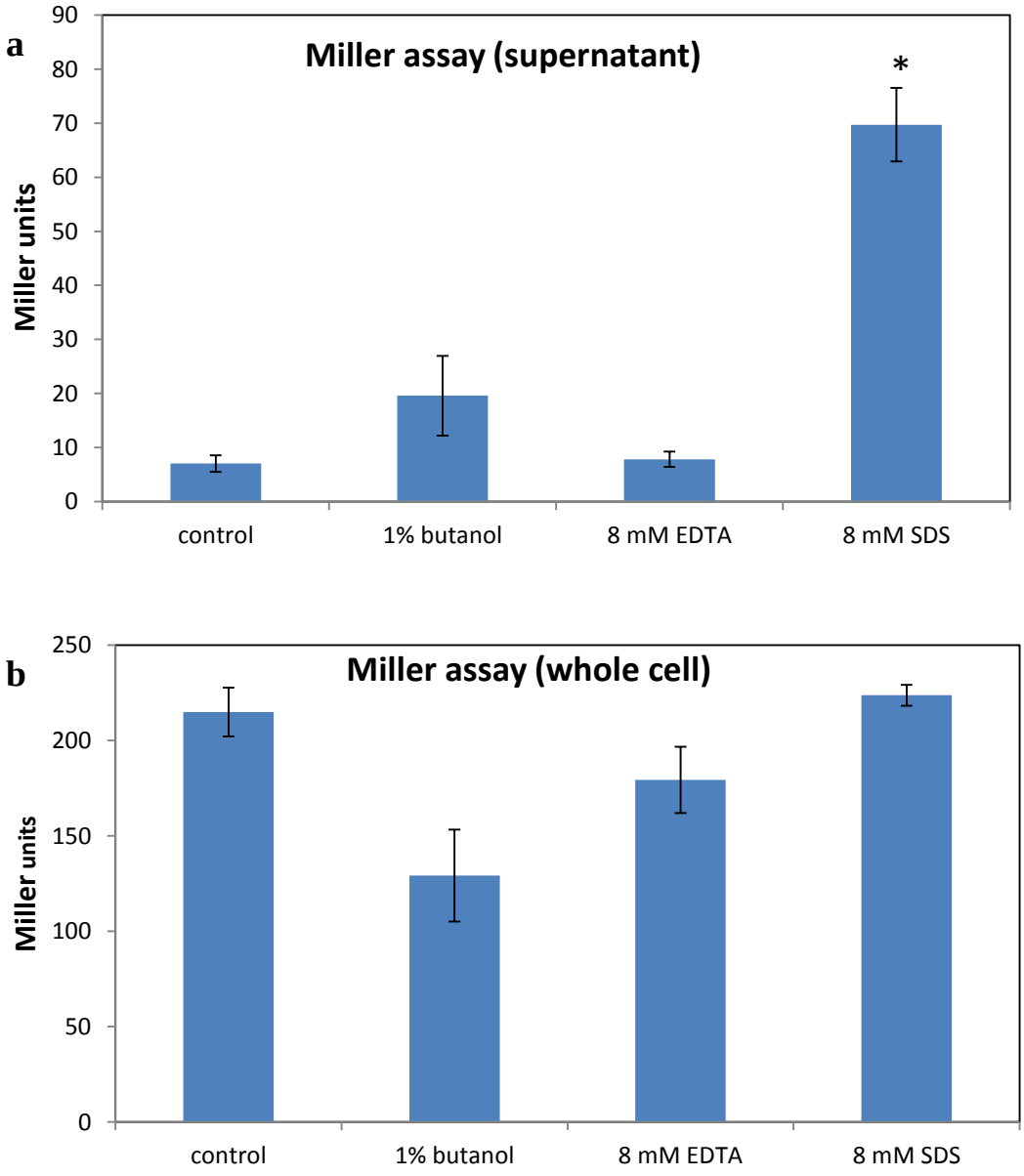


Figure 4.3: Effect of *n*-butanol on the inner membrane of *E. coli*.

β-galactosidase activity *E. coli* MG1655 cells treated with butanol, EDTA or SDS was measured using a) culture supernatants and b) whole cells. Control experiments were carried out with SDS and EDTA. Experiments were done in triplicate. Standard errors are shown with the error bars. Asterisk signifies statistical significance ($p < 0.05$).

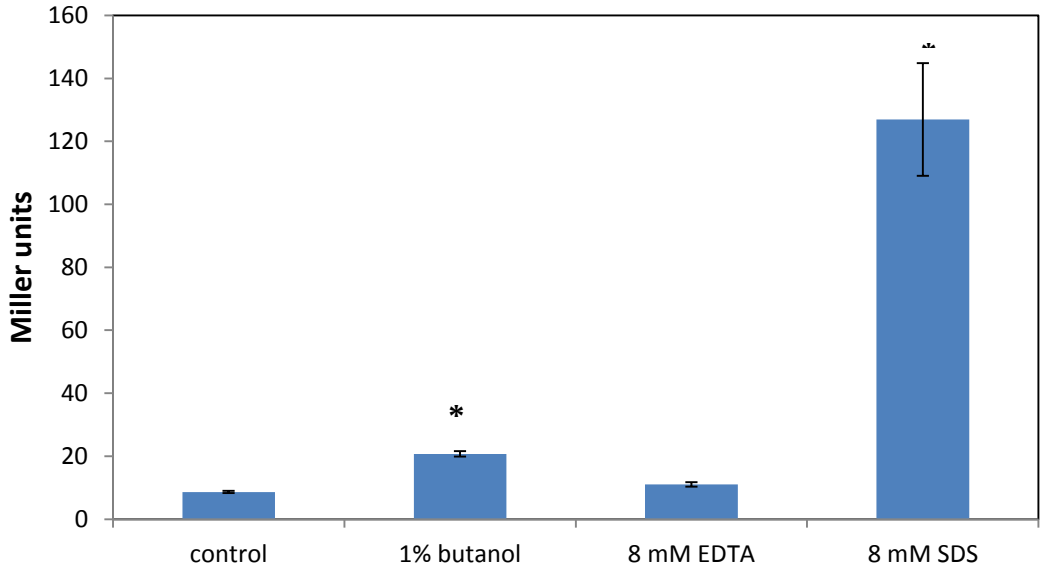


Figure 4.4: Effect of *n*-butanol on the inner membrane.

E. coli cells were exposed to *n*-butanol for 2 hr and the supernatant of the cultures were assayed for β-galactosidase activity. Experiments were done in triplicate. Standard errors are shown with the error bars. Asterisk signifies statistical significance ($p < 0.05$).

Another factor that could explain the relatively low β-galactosidase activity in the supernatants of cultures exposed to *n*-butanol is the size of the enzyme. β-galactosidase is a huge homotetramer of size 464 kDa (Jacobson *et al*., 1994) which might not be able to leak out of the cell unless the cell membrane is significantly destroyed. Therefore, another assay was employed to detect inner membrane damage. Cells were transformed with a smaller protein, RFP (dsRed), with a molecular size of 28 kDa (Baird *et al*., 2000) and exposed to increasing concentrations of *n*-butanol. Red fluorescence was detected in the supernatants of cultures exposed to *n*-butanol with the intensity of fluorescence increasing correspondingly with the amount of *n*-butanol added (Figure 4.5).

It is also worth noting that even though cells exposed to SDS had a much higher amount of alkaline phosphatase activity than those exposed to *n*- butanol, the cells grew better in SDS than they did in *n*-butanol (Figure 4.6).

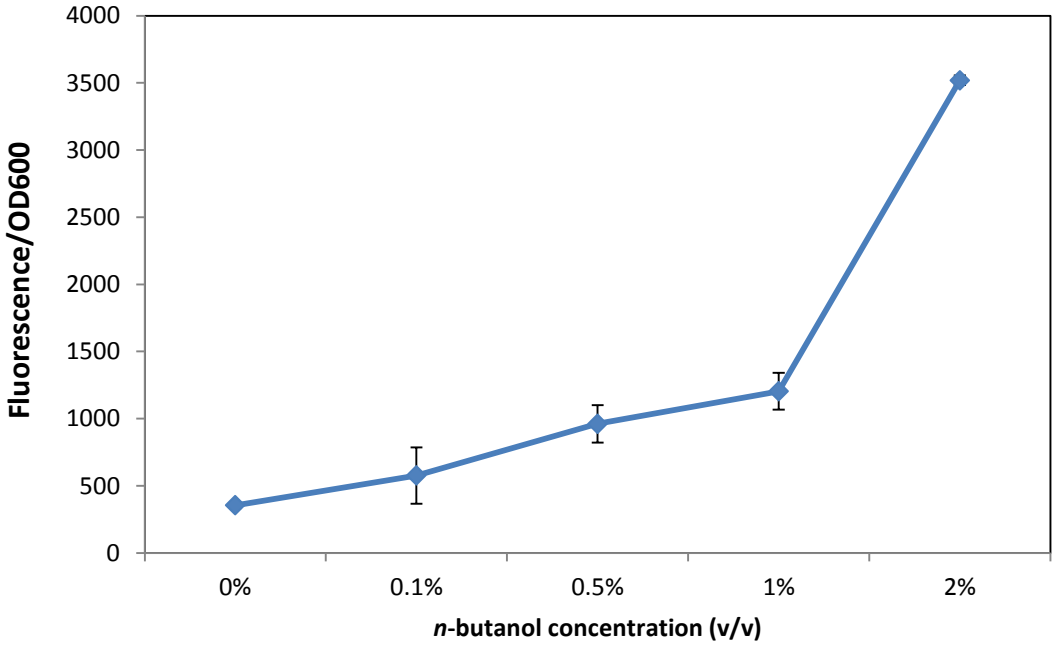


Figure 4.5: Effect of *n*-butanol on the inner membrane of *E. coli*.

Cells transformed with RFP were treated with increasing concentrations of *n*-butanol and red fluorescence was measured in the supernatant of the cultures.

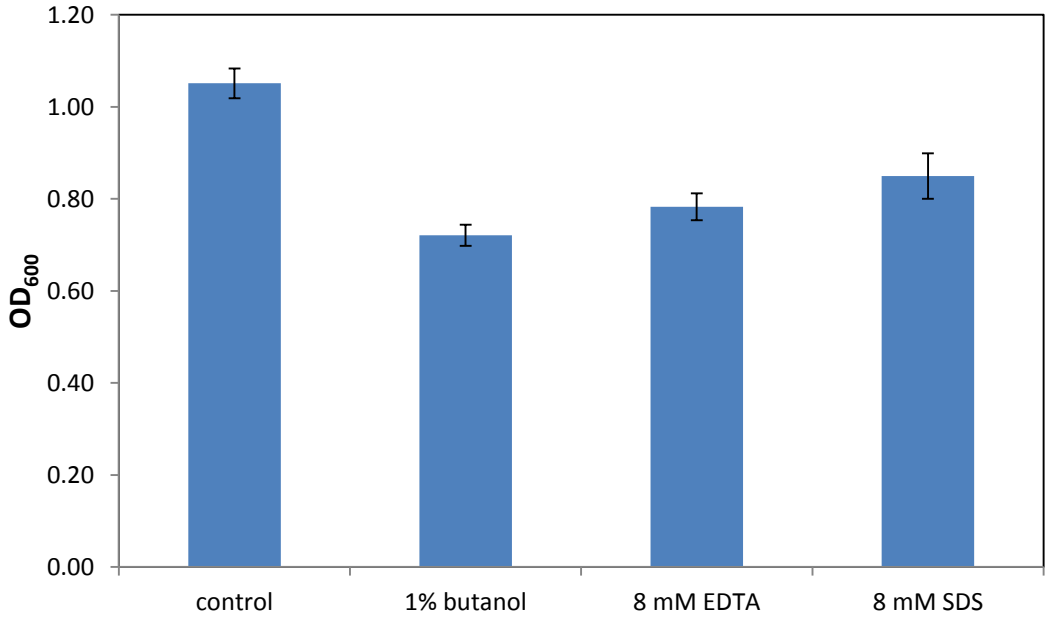


Figure 4.6: Growth inhibition.

The effect of *n*-butanol, EDTA and SDS on the growth (OD_{600}) of *E. coli* was measured after cells were exposed to these chemicals for 1 hr prior to being used for the enzyme assays. Experiments were done in triplicate. Standard errors are shown with the error bars.

4.2.3 Development of a bioreporting system to detect outer membrane damage

The effect of *n*-butanol on the outer membrane of *E. coli* was further investigated with a bioreporting system. The bioreporting system was designed by placing a promoter that is responsive to membrane damage upstream of an output reporter (Figure 4.7). In this experiment the *lptA* promoter was chosen and was placed upstream of RFP (DsRed). The rationale for choosing the *lptA* promoter was because it is induced when there is the need for the cell to transport lipopolysaccharides (LPS) to the outer membrane (Sperandeo *et al*., 2003). It has also been shown to be upregulated during cell stress possibly as a result of membrane damage (Reyes *et al*., 2012).

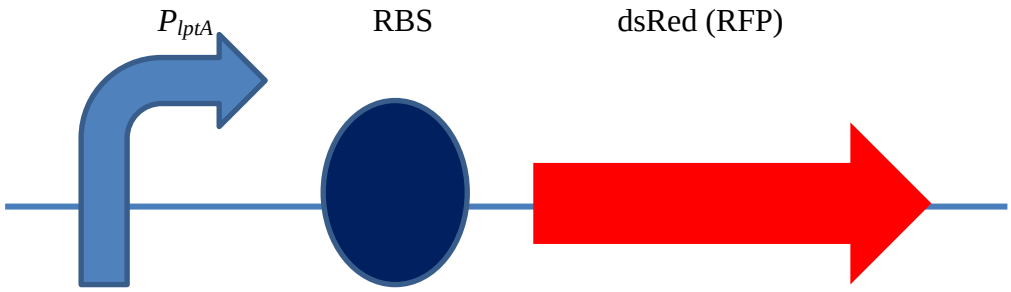


Figure 4.7: Architecture of the *lptA* bioreporter.

The *lptA* promoter (P_{lptA}) was placed upstream of the red fluorescence protein (dsRed) and a ribosome binding site (RBS) designated BBa_B0034 in the Registry of Biological Parts (www.partsregistry.org)

E. coli cells transformed with this construct on pSB1C3 were exposed to increasing concentrations of *n*-butanol just when they reached the exponential phase (OD_{600} = 0.3) for 2 hr. Induction of the *lptA* promoter was determined by measuring red fluorescence. The cell densities (OD_{600}) were also measured. Since the amount of fluorescence is dependent on the cell density, a ratio of fluorescence to OD_{600} at each time point was used to determine induction of the *lptA* promoter. Figure 4.8a shows that the fluorescence output is proportional to the concentration of *n*-butanol in the media since fluorescence increased over time when the cells were exposed to increasing concentrations of *n*-butanol. Even though the amount of fluorescence seems identical for all concentrations, the cell growth (OD_{600}) shows that higher concentrations of *n*-butanol impair growth (4.8b). The Fluorescence/OD_{600} measurements indicate that the output of fluorescence is proportional to the concentration of *n*-butanol added (Figure 4.8c).

A control experiment was carried out with increasing concentrations of EDTA. When the concentration of EDTA was increased, there was a corresponding increase in the fluorescence output signifying membrane stress (Figure 4.9).

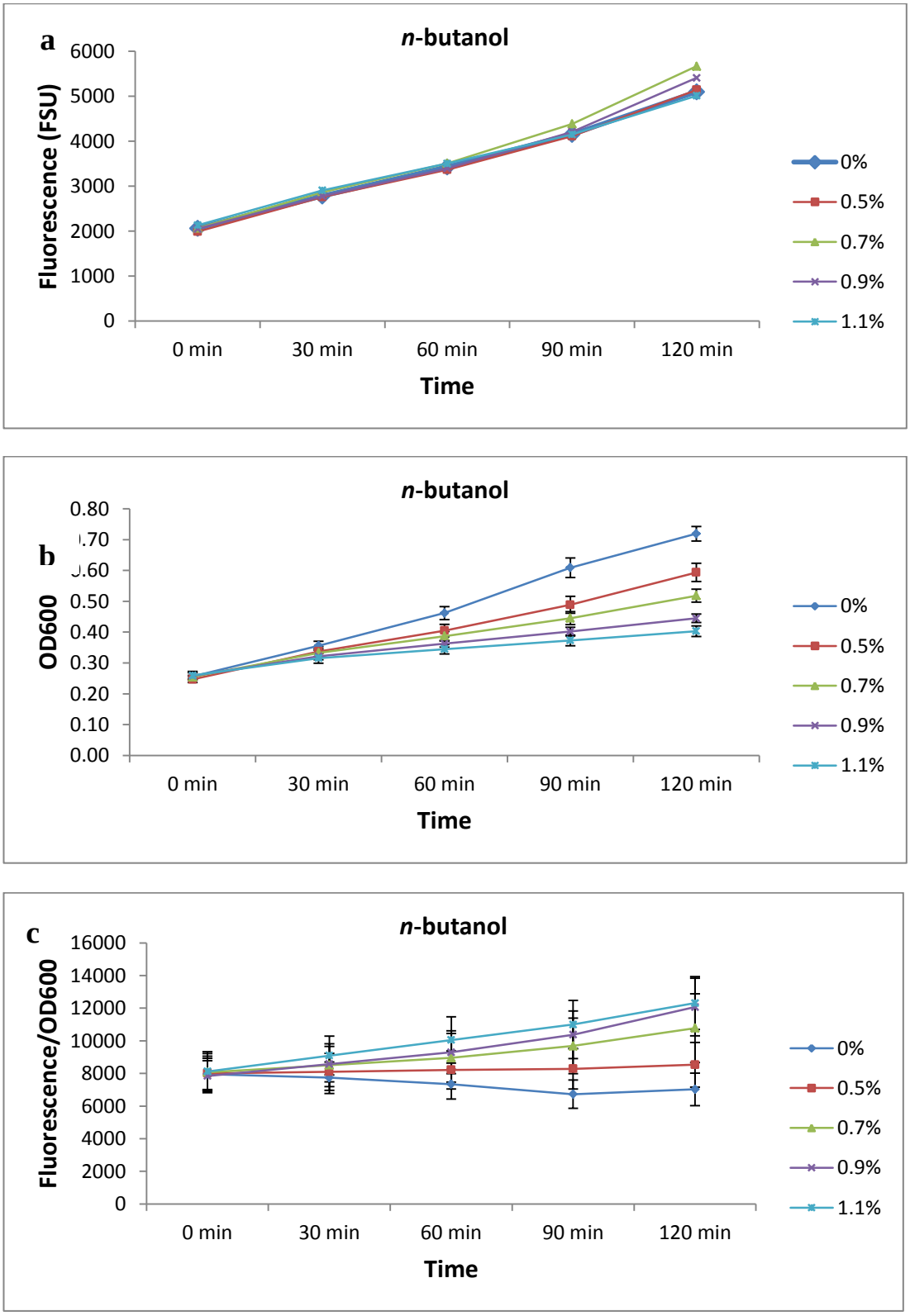


Figure 4.8: Characterisation of the *lptA* bioreporter.
The *lptA* bioreporter was characterised with various *n*-butanol concentrations. The fluorescence (a), OD_{600} (b) and fluorescence/OD_{600} (c) measurements were used to determine the response of the bioreporter to *n*-butanol. Experiments were done in triplicate. Standard errors are shown with the error bars.

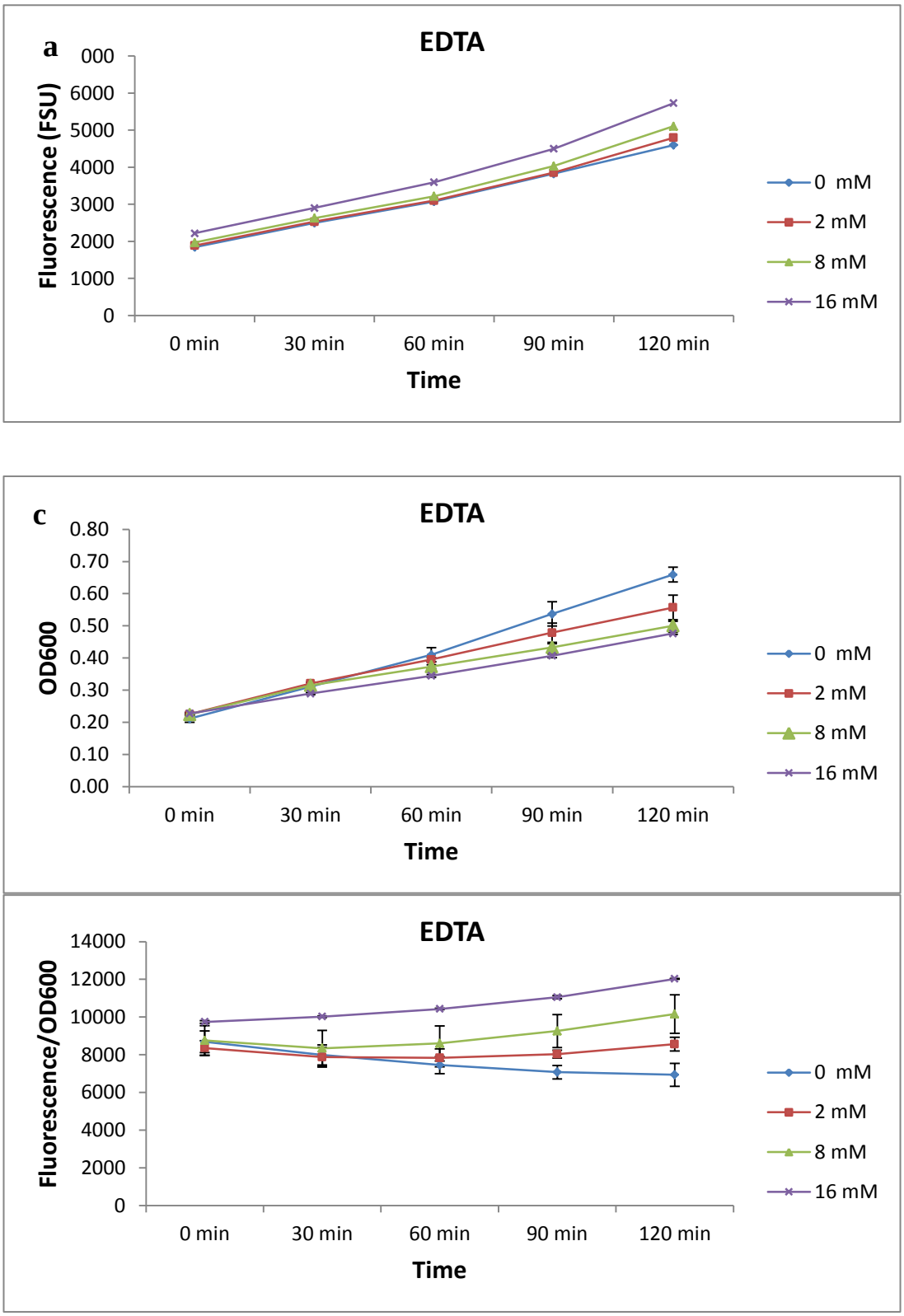


Figure 4.9: Effect of EDTA on the *lptA* bioreporter.
The effect of different concentrations of EDTA on the induction of the *lptA* bioreporter was studied in a control experiment. The fluorescence (a), OD_{600} (b) and fluorescence/OD_{600} (c) readings were used to determine the response of the bioreporter to EDTA. Experiments were done in triplicate. Standard errors are shown with the error bars.

4.2.4 LPS release from the outer membrane

Next, to test the hypothesis that *n*-butanol damaged the outer membrane by removing the lipopolysaccharides on the outer membrane, cells were grown till they reached the exponential phase after which they were treated with *n*-butanol for 1 hr and the amount of LPS released into the supernatant was assayed. It was observed that the amount of LPS released into the supernatant increased with increasing concentrations of *n*-butanol (Figure 4.10a). A similar trend was observed when cells were treated with increasing concentrations of EDTA (Figure 4.10b). Comparing the amount of LPS released into the supernatant during *n*-butanol treatment to a 'no solvent' control in Figure 4.10c, it was observed that *n*-butanol-treated cells had a significantly higher amount of LPS released ($p = 0.012$). The amount of released LPS was much higher in the EDTA control experiment ($p = 0.0001$).

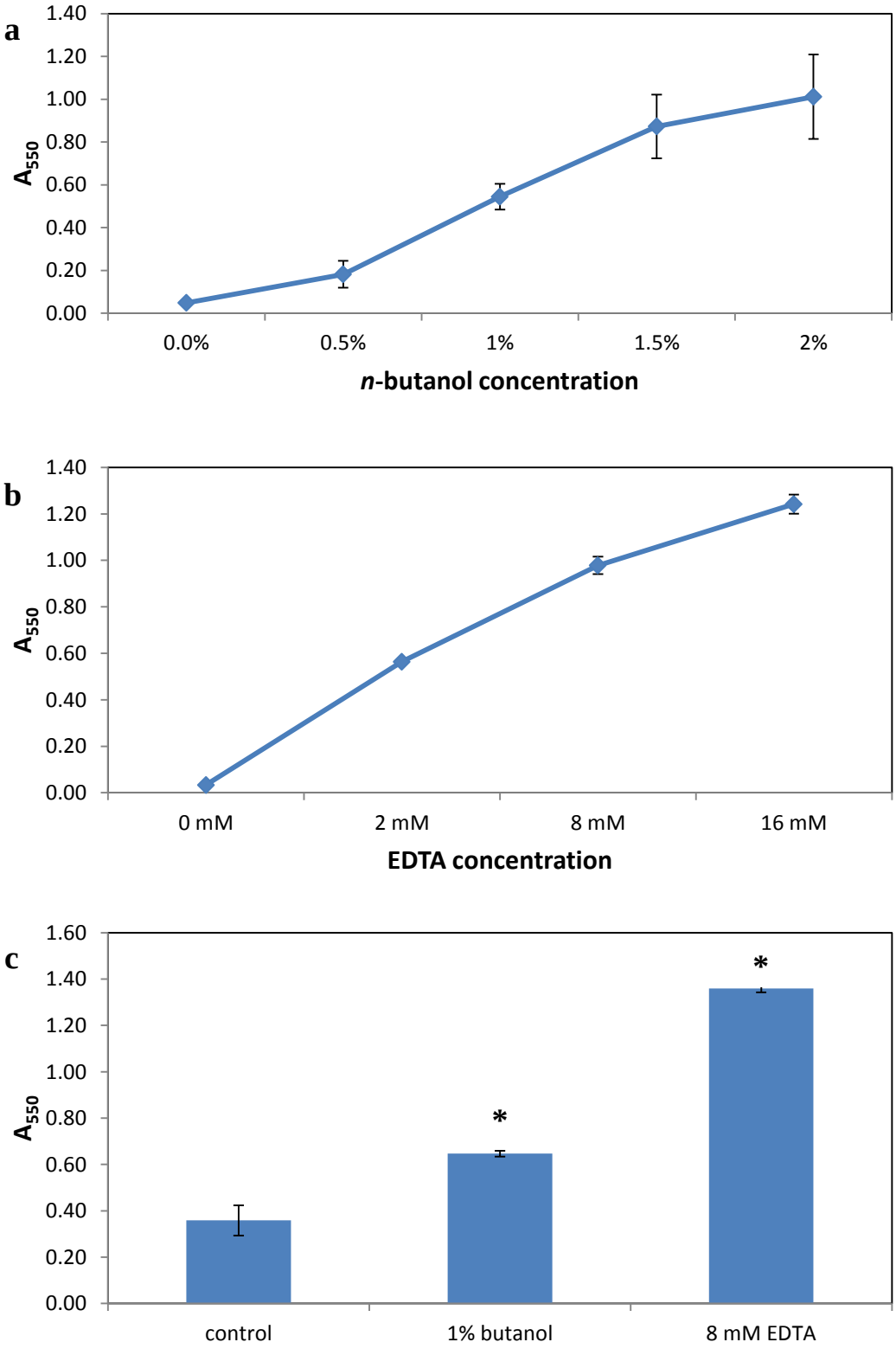


Figure 4.10: Effect of *n*-butanol on the LPS.
Removal of LPS from the outer membrane into the supernatant by *n*-butanol was determined with the purpald assay (a, c). EDTA was used in a control experiment (b, c). Experiments were done in triplicate. Standard errors are shown with the error bars. Asterisk signifies statistical significance ($p < 0.05$).

4.2.5 Role of divalent cations on *n*-butanol tolerance

The effect of two divalent cations (Mg^{2+} and Ca^{2+}) on the membrane and their contribution to *n*-butanol tolerance was investigated. *E. coli* cells were grown in LB supplemented with 0.5% *n*-butanol and increasing concentrations of Mg^{2+} (0 mM, 5 mM, 10 mM, 15 mM and 20 mM) or Ca^{2+} ions (0 mM, 1.5 mM, 3 mM, 4.5 mM and 6 mM). In the presence of 10 mM Mg^{2+}, the cells grew slightly better in *n*-butanol than at the other concentrations of magnesium tested (Figure 4.11). Higher concentrations of magnesium proved to be slightly more toxic to the cells. None of the concentrations of Ca^{2+} tested increased tolerance to *n*-butanol (Figure 4.12).

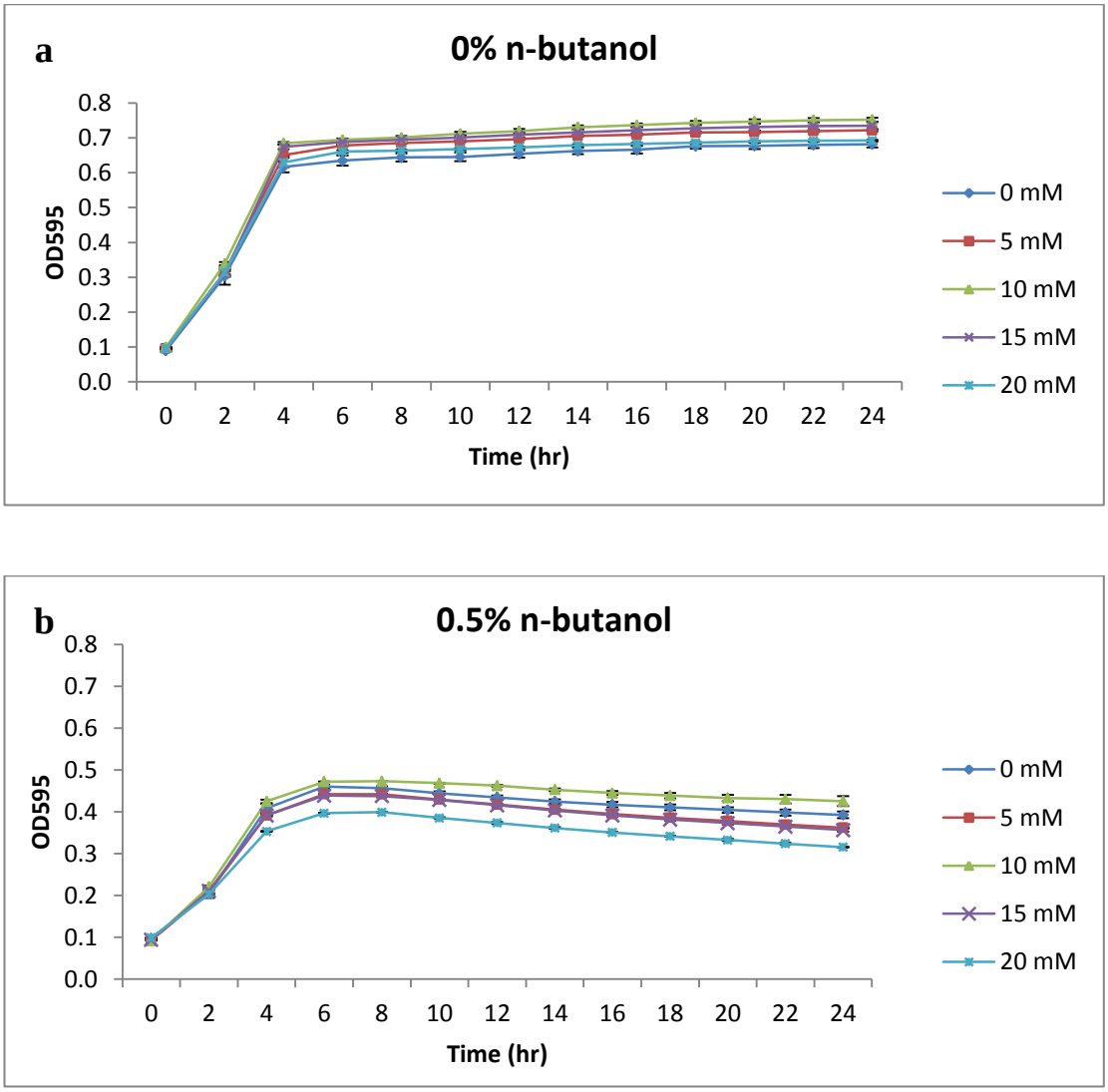


Figure 4.11: Effect of magnesium ions on *n*-butanol tolerance.

Overnight cultures of *E. coli* MG1655 were inoculated in fresh LB in the presence (a) or absence of 0.5% *n*-butanol (b) supplemented with various concentrations of $MgSO_4$. Cell growth was determined at 595 nm. Error bars indicate standard error of three biological replicates.

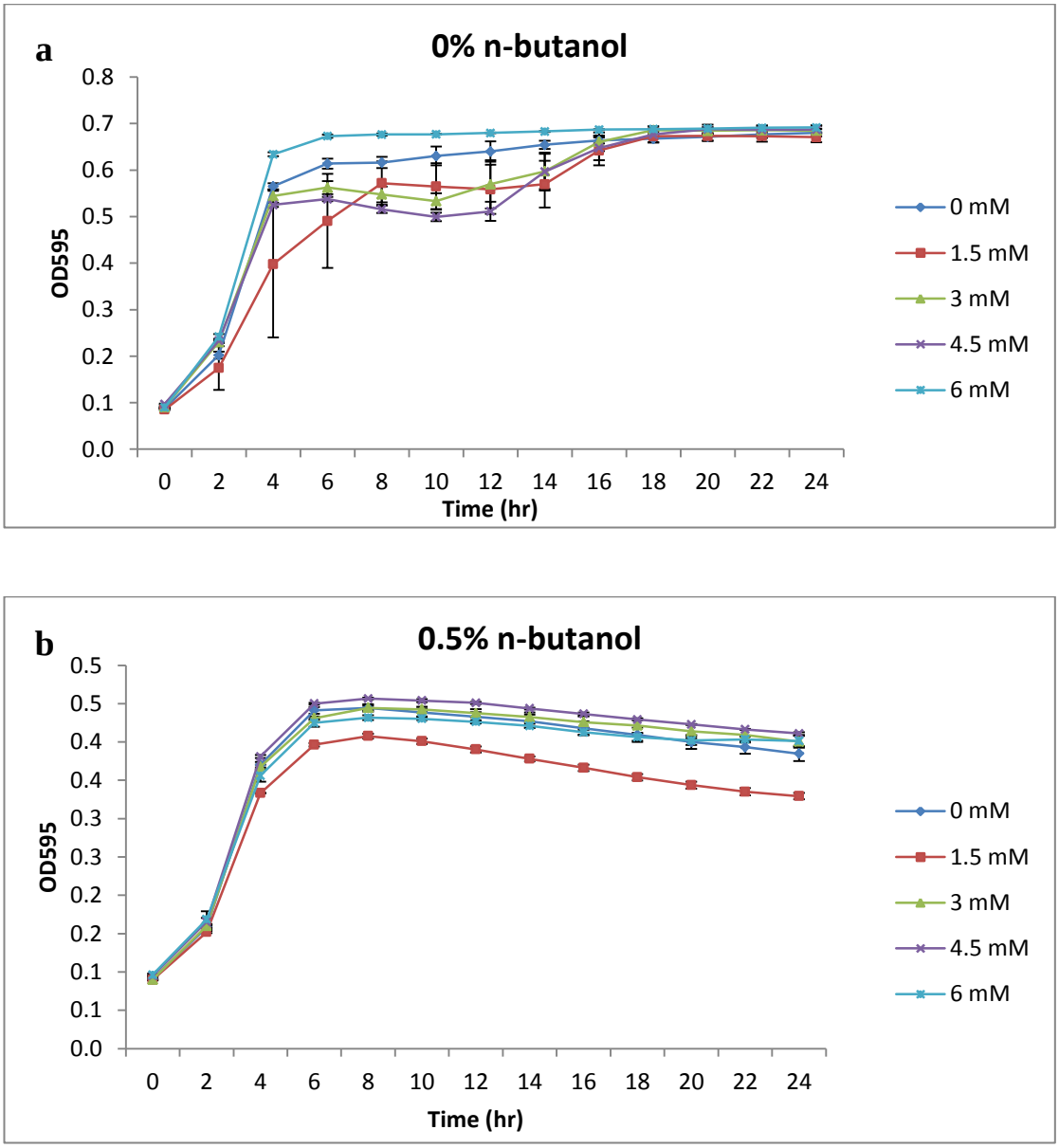


Figure 4.12: Effect of calcium ions on *n*-butanol tolerance.

Overnight cultures of *E. coli* MG1655 were inoculated in fresh LB in the presence (a) or absence of 0.5% *n*-butanol (b) supplemented with various concentrations of $CaCl_2$. OD_{595} readings were taken to determine cell growth. Error bars indicate standard error of three biological replicates.

The alkaline phosphatase assay was used to determine the protective effect of Mg^{2+} ions on the outer membrane as described earlier. The alkaline phosphatase activity in the supernatant of cultures containing *n*-butanol was higher than the control cultures without *n*-butanol signifying outer membrane damage (Figure 4.13). However, cultures containing *n*-butanol and supplemented with 10 mM Mg^{2+} had a lower alkaline phosphatase activity in the supernatant (p = 0.016) than those containing *n*-butanol but not supplemented with Mg^{2+} implying that the outer membrane of the latter was more leaky than the former.

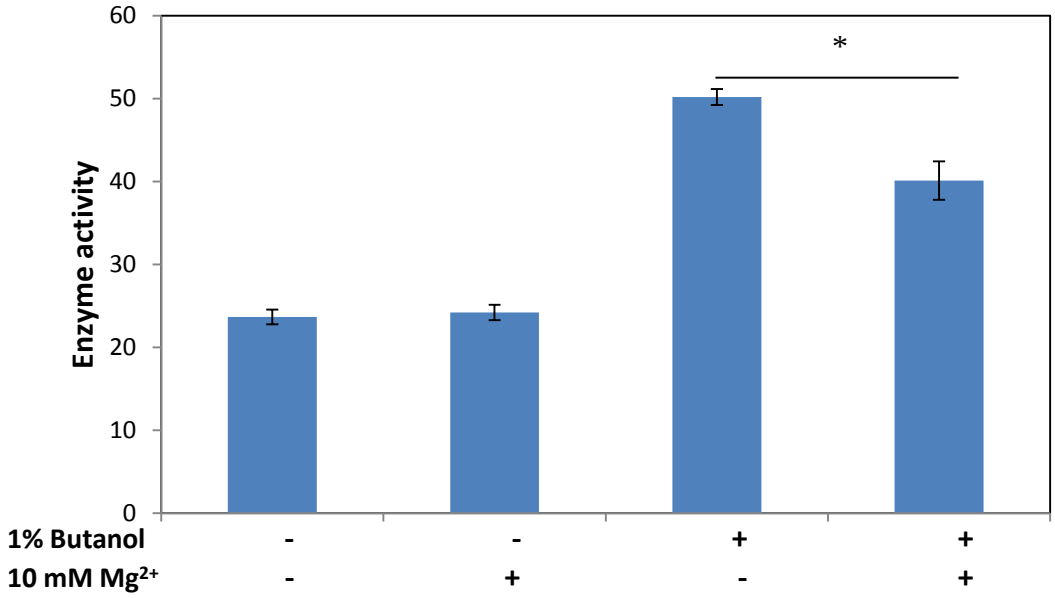


Figure 4.13: Protective effect of magnesium ions on the outer membrane.
Using the alkaline phosphatase assay the effect of magnesium in reducing membrane damage when cells were exposed to *n*-butanol was determined. Asterisk signifies statistical significance ($p < 0.05$).

4.2.6 Morphological characterisation of the effect of *n*-butanol

To determine the effect of *n*-butanol on the cell shape of *E. coli*, different concentrations of *n*-butanol was added to exponential phase cultures to a final concentration of 0.5% and 1%. In a control experiment, no *n*-butanol was added to the cultures. The cells, transformed with *egfp*, were incubated for 1 hr in *n*-butanol and were observed under a fluorescent microscope. The cells in the control cultures and those containing 0.5% *n*-butanol maintained their rod-like shape and looked normal. However, those exposed to 1% *n*-butanol were elongated and filamented (Figure 4.14). Another observation worth noting was that it appeared the 1% cultures had fewer cells per µl of culture compared to the control cultures and those containing 0.5% *n*-butanol.

The effect of very high concentrations of *n*-butanol (2%, 5% and 10% v/v) on the cell was also investigated. Since these concentrations are extremely toxic to *E. coli*, it was expected that cell lysis would occur soon after the addition of solvent. Therefore, a time-lapse experiment using the fluorescent microscope was used to investigate the various stages the cell went through before lysis occurred. To do this, cells fixed in a trench made on a microscope slide (see Materials and Methods) were flushed with the different concentrations of *n*-butanol and observed under the microscope. For cells exposed to 2% *n*-butanol there was no lysis observed in the first 30 min even though the cells became elongated. Those exposed to 5% and 10% *n*-butanol went through two stages prior to lysing. The cells became elongated which was followed by the formation of blebs on the cells and then, lysis (Figure 4.15). The bleb formation was short-lived and the whole event right from the addition of the solvent

to cell lysis lasted 10 min. The blebs also fluoresced green implying that it was filled with contents from the cytoplasm of the cell.

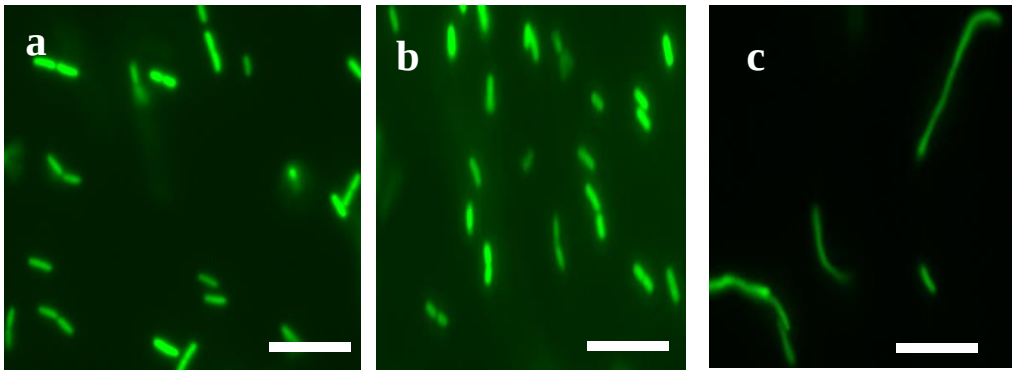


Figure 4.14: The effect of low concentrations of butanol on cell shape.
Fluorescent images obtained for cultures exposed to (a) 0%, (b) 0.5% and (c) 1% *n*-butanol for 1 hr showed the effect of the solvent on the cells.
Scale bar represents 6 µm.

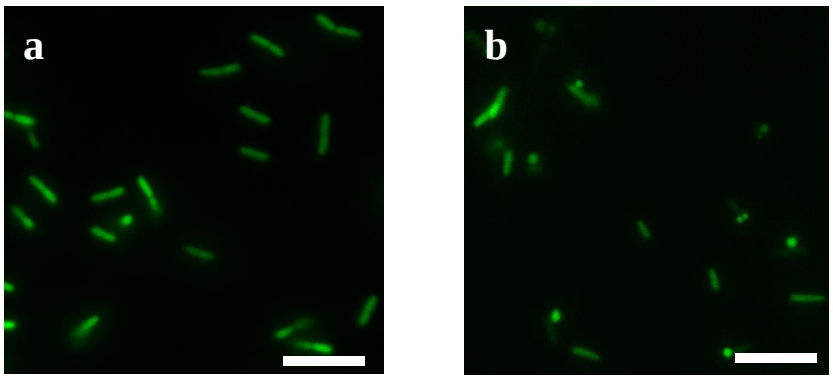


Figure 4.15: Morphological characterisation of cells exposed to 5% *n*-butanol.
Fluorescent images taken of single cells shows that cells flushed with very high concentrations of *n*-butanol become (a) elongated, (b) form blebs and lyse. Scale bar represents 6 µm.

4.3 Discussion

n-butanol has gained a lot attention as a superior biofuel alcohol to ethanol. However, since it is more hydrophobic than ethanol it is also more toxic to microbial hosts used to produce it. A lot of work has focussed on the effect of ethanol on the cell membrane (Ingram and Vreeland, 1980; Huffer *et al*., 2011) but there remains a gaping gap in research highlighting the effect of butanol on the cell membrane. The exact mechanism by which butanol crosses the cell membrane into the cell and the resulting effect on the membrane during this process is not known although butanol has been reported to intercalate into the cell membrane resulting in increased fluidity of the membrane (Ingram, 1986). The aim of this chapter was to investigate the actual effect of *n*-butanol on the membrane integrity of *E. coli* and to probe possible ways to maintain this membrane integrity by supplementing the culture media with divalent cations.

In this study, it was observed that both outer and inner membranes of *E. coli* were weakened when cells were exposed to *n*-butanol resulting in leakage of periplasmic and cytosolic enzymes, among others, respectively. The effect of short chain alcohols like ethanol on the membrane has been widely studied. Ethanol has been shown to result in leakage of the plasma membrane resulting in adverse effects on the growth of bacteria (Ingram, 1990). In yeasts, it has been shown that ethanol interacts with the hydrophobic regions of the cytoplasmic membrane and denatures the proteins involved in the uptake of sugars into the cell (Carlsen *et al*., 1991). Very little effort has gone into investigating the effect of *n*-butanol on the membranes. However, it has been reported that butanol, unlike shorter chain alcohols such as ethanol and methanol, interacts more with the hydrophobic regions of the cell membrane (Ly and Longo, 2004). Leakage of cytosolic and periplasmic enzymes from cells exposed to

n-butanol was detected in this study. Butanol, being a longer chain alcohol permeates and intercalates further into the hydrophobic phospholipid layer of the cell membrane (Ly and Longo, 2004). This interaction of *n*-butanol with membrane breaks the hydrogen bonds between the lipid tails of the phospholipids and possibly alters the fluidity of the membrane as well as making the membrane leaky. Also emerging from this work is the fact that a high amount of membrane leakage induced by SDS does not inhibit growth as much as *n*-butanol does even though the latter induced less membrane leakage. This shows that weakening the membrane is not the sole cause of butanol-induced growth inhibition. There are other damaging effects of *n*-butanol on the cell which include protein denaturation (Gonzalez-Ramos *et al*, 2013), among others (Rutherford *et al*., 2010). Increase in membrane fluidity has also been reported as just one of the causes of growth inhibition by alcohols and not the sole cause (Huffer *et al*., 2011).

Most hydrophobic compounds are prevented from traversing the cell membrane by the hydrophilic lipopolysaccharides (LPS) on the outer membrane. Since *n*-butanol is hydrophobic, it was hypothesised in this study that for *n*-butanol to reach the lipid bilayer it must be able to cross the LPS barrier by some mechanism. To show this, the effect of *n*-butanol on the outer membrane was further investigated using a bioreporting system composed of the *lptA* promoter attached to an output signal (RFP). This promoter drives the expression of the LptA protein in the periplasm which plays an essential role in the transport of LPS molecules to the outer leaflet of the outer membrane during LPS biosynthesis and when the outer membrane is threatened (Sperandeo *et al*., 2003). Therefore, it would be expected that the *lptA* promoter will be induced when the outer membrane is stressed. Reyes *et al*., (2012) found that the *lptA* gene was upregulated when *E. coli* was exposed to *n*-butanol.

Induction of this promoter increases when cells are exposed to EDTA, a known outer membrane-damaging agent. A similar effect was observed with increasing concentrations of *n*-butanol confirming the damaging effect of *n*-butanol on the outer membrane. Prior to that report by Reyes *et al*., (2012), this gene had not been implicated in alcohol stress. This confirms the possible involvement of the LPS during *n*-butanol stress.

Gram negative cells possess an outer membrane which has LPS molecules predominantly on the outer leaflet creating a hydrophilic environment preventing the entry of hydrophobic compounds into the cell. LPS mutants or cells with LPS removed, however, are more susceptible to hydrophobic antibiotics as these hydrophobic compounds are able to freely permeate the membrane (Leive, 1974). It was hypothesised in this work that *n*-butanol destroyed the hydrophilic barrier on the outer membrane of *E. coli* by removing the LPS molecules on the outer leaflet of the cell membrane and rendered the membrane permeable. Therefore its ability to remove LPS molecules from the outer membrane was examined. LPS release was observed when cells were treated with *n*-butanol. Similarly, EDTA which is known to weaken the outer membrane and render it more permeable to hydrophobic compounds (Alakomi *et al*., 2006; Schnaitman *et al*., 1971) induces LPS release from the outer membrane by chelating the magnesium ions bridging the LPS molecules as reported in other studies (Hua *et al*., 2007). In a related study, *Pseudomonas aeruginosa* cells treated with rhamnolipid to release the outer membrane LPS were found to be more permeable to hexadecane (Al-Tahhan *et al*., 2000). Furthermore, the upregulation of certain LPS biosynthesis genes during butanol challenge confirms the involvement of the LPS in butanol stress response (Reyes *et al*., 2012). Increasing the LPS content on the cell during such stress makes

the cell surface less hydrophobic and prevents hydrophobic compounds from entering the cell. These results therefore suggest one possible mechanism of *n*-butanol entry into the cell. On contact with the cell, *n*-butanol breaks the hydrophilic LPS barrier by releasing the LPS molecules possibly by removing or interacting with the divalent ion bridges holding the LPS molecules together. This then exposes the hydrophobic lipid layer of the outer membrane for butanol to interact with and cross. As it traverses the periplasm, it is able to insert itself into the hydrophobic inner membrane as well. The exact effect of *n*-butanol on the LPS-LPS bridges has not been studied and requires further investigation. It is also likely, however, that the solvent might be able to cross the membrane into the cell by other mechanisms such as through membrane porins. This possibility should not be ruled out and requires further investigation.

Work done in this chapter also looks at how different divalent ions can increase tolerance to butanol by fortifying the LPS bridges in a bid to prevent butanol from breaking this barrier to enter the cell. As mentioned earlier, magnesium is an important metal required for maintaining the interactions between the LPS molecules on the outer membrane. Its removal results in LPS release which also results in increased permeability of the cell membrane to hydrophobic compounds. The results presented in this work show that supplementing the culture media with magnesium ions increases tolerance to butanol confirming an earlier report (Inoue *et al*., 1991). Also, in *Pseudomonas putida*, Mg^{2+} ions have been implicated in solvent tolerance (Ramos *et al*., 1995). However, high concentrations of magnesium (>10mM) seem to be toxic to the cells. This suggests that supplementing the growth media of *E.coli* with the right amount of magnesium increases *n*-butanol tolerance probably by preventing the interaction of *n*-butanol with the LPS-LPS interaction. In another

study, the ability of EDTA to permeabilise the membrane was not completely abolished by the addition of excess amounts of $MgCl_2$ (Alakomi *et al*., 2006) showing that even though Mg^{2+} ions have a protective effect on the outer membrane, this effect is just slight. Damage done by *n*-butanol to the outer membrane was also reduced when the culture was supplemented with Mg^{2+}. Ca^{2+} did not increase tolerance to *n*-butanol contrary to a previous study (Seregina *et al*., 2012). Similarly, it has been shown that Mg^{2+} increases solvent tolerance in *P. putida* even though other divalent cations including Ca^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+} did not exert any beneficial effect on solvent tolerance in the same organism (Ramos *et al*., 1995).

Using fluorescence microscopy, the changes in the cell shape of *E. coli* exposed to *n*-butanol was studied which gave further information on the effect of the solvent on the cell envelope. Cell elongation during *n*-butanol stress as observed in the present study might be due to an arrest of cell division suggesting that *n*-butanol has a negative effect on the cell's DNA either directly or indirectly. Two things might have happened during the solvent stress - either key proteins and enzymes involved in DNA synthesis were inhibited by butanol or reactive oxygen species generated by *n*-butanol (Rutherford *et al*., 2010) may have damaged the DNA. When exposed to 5% *n*-butanol, not only did the cells become elongated but they also formed blebs similar to what was observed when *E. coli* was treated with beta-lactam antibiotics (Yao *et al*., 2012). Beta-lactam antibiotics prevent cross-linking of the peptidoglycan layer in the periplasm. Therefore, cells treated with such antibiotics will have a damaged peptidoglycan causing the cytosolic content to bulge out of the cell. The stability of the bulge or bleb depends on the strength of the inner and outer membranes (Yao *et al*., 2012). In this work, the blebs formed on the *E. coli* cells suggested that very high concentrations of *n*-butanol damaged the peptidoglycan. This effect of *n*-butanol on

the peptidoglycan has not been previously reported to the best of my knowledge even though some transcriptional studies have reported the upregulation of genes involved in peptidoglycan biosynthesis during isobutanol stress (Atsumi *et al*., 2010). Ethanol, but not hexanol, has been shown to alter cross-linking of peptidoglycan in *E. coli* (Ingram and Vreeland, 1980). It will be interesting, therefore, to investigate further the effect of *n*-butanol on the peptidoglycan layer. Also, the weakening effect of *n*-butanol on the membrane was confirmed in this experiment by the short life and quick lysis of the blebs since a strong and intact membrane is required to maintain the stability of these bulges and prevent them from bursting.

In summary, results presented in this chapter show that *n*-butanol has a damaging effect on the cell membrane and peptidoglycan layer of *E. coli*. Butanol overcomes the hydrophilic LPS barrier and then weakens the cell membrane of *E. coli* by intercalating in it. This results in leakage of periplasmic and cytosolic contents. These results provide several cues that can serve as a guide to rationally engineer solvent tolerant microbial hosts. One strategy, among others, that can be employed is to fortify the outer membrane by supplementing liquid cultures with the appropriate amount of magnesium. It is also important to tackle the problem of solvent toxicity at both membrane and cytosolic levels.

Chapter 5: Increasing *E. coli n*-butanol tolerance by *cis-trans* isomerisation of membrane fatty acids

5.1 Introduction

Toxic compounds such as *n*-butanol interfere with membrane fluidity and cause cellular contents to leak out of the cell as was shown in the previous chapter of this thesis. When exposed to harsh environments, micro-organisms employ several smart strategies to protect themselves from such stress. One of those strategies is ensuring that the membrane is well-fortified such that its integrity is not compromised.

In the presence of sub-lethal concentrations of toxic compounds, *E. coli* alters the composition of the phospholipids in its membrane bilayer to compensate for any damaging effects of the toxic compound on the membrane. The cell can achieve this by increasing the ratio of saturated fatty acids to unsaturated fatty acids. This change in membrane composition has been reported to alter membrane fluidity when some strains of *Pseudomonas* were exposed to organic solvents (Ramos *et al*., 2002). In *E. coli*, a *ΔfadR* mutant was shown to increase tolerance to *n*-hexane by preventing the solvent from entering the cell (Oh *et al*., 2012). Fatty acid analysis of the membrane of this mutant *E .coli* revealed elevated levels of saturated fatty acids confirming a link between high levels of saturated fatty acid in the membrane and tolerance to organic solvents. Some other bacteria modify their membrane to protect it against environmental stress by increasing chain length of the fatty acids in the membrane (Kanno *et al*., 2013). The presence of long chain fatty acids in *Lactobacillus heterohiochii* has been reported to be responsible for its tolerance to ethanol (Uchida, 1974). Furthermore, conversion of unsaturated fatty acids to cyclopropane fatty acids

in *E .coli* and some other organisms has been reported as another mechanism by which these organisms modify their membranes (Ingram, 1977; Kanno *et al*., 2013). Indeed, overexpression of the *cfa* gene which encodes the Cyclopropane synthase responsible for this conversion has been shown to increase tolerance to butanol, acid stress and osmotic stress in *E. coli* and *Clostridium acetobutylicum* (Zhao *et al*., 2003)

Previous work by Rühl *et al*., (2009) has shown that *Pseudomonas putida*, a gram negative bacterium, is able to resist high concentrations of butanol and other organic solvents. This natural ability to resist toxic compounds is due to the presence of a *cis-trans* isomerase (CTI) which converts *cis* unsaturated fatty acids in the membrane to *trans* unsaturated fatty acids (Heipeiper *et al*., 1992) resulting in a reduction in membrane fluidity. *Cis* unsaturated fatty acids form a bend in the fatty acid chain making the membrane more fluid whereas the *trans* form straightens the chain and increases the rigidity of the membrane. This process has been described as a short-term effect allowing the cells enough time to quickly put in place other mechanisms to overcome the stress (Ramos *et al*., 2002). CTI is a cytochrome c-type protein which is constitutively expressed in *Pseudomonas* species (Holtwick *et al*., 1999). However, its enzyme activity is activated in the presence of organic solvents and elevated temperatures (Pedrotta and Witholt 1999).

Although CTI has been previously expressed in *E. coli* (Holtwick *et al*., 1999), its ability to increase *n*-butanol tolerance in *E. coli* by modifying the membrane has not been fully explored. In this chapter, the possibility of tackling the problem of *n*-butanol toxicity at the membrane level by expressing CTI in *E. coli* will be investigated. Using fluorescence microscopy and a fluorescence-based assay, the

protective effect of CTI on the membrane during *n*-butanol stress will be investigated. Finally, synergistic interactions between the *cti* gene and a gene encoding the molecular chaperone, Prefoldin, from *Pyrococcus horikoshii* which was shown to increase *E. coli* tolerance to *n*-butanol in Chapter 3 will be explored.

5.2 Results

5.2.1 Bioinformatics search for CTI homologues in *E. coli*

To identify possible CTI homologues in *E. coli*, the *E. coli* genome at the NR (non-redundant) database was searched using the Protein BLAST (Basic Local Alignment Search Tool - blastp) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In a gapped search when the CTI sequence from *Pseudomonas putida* DOT-T1E was blasted against the *E. coli* genome, fourteen BLAST hits were obtained. The best hit with an *E-value* of 0.18 was a liprotein, glycosyl hydrolase homolog (NCBI Accession number NP_416008.1) which meant that there was an 18% probability that this alignment is purely by chance given the size of protein aligned. Therefore, no putative CTI homologues were identified from the search.

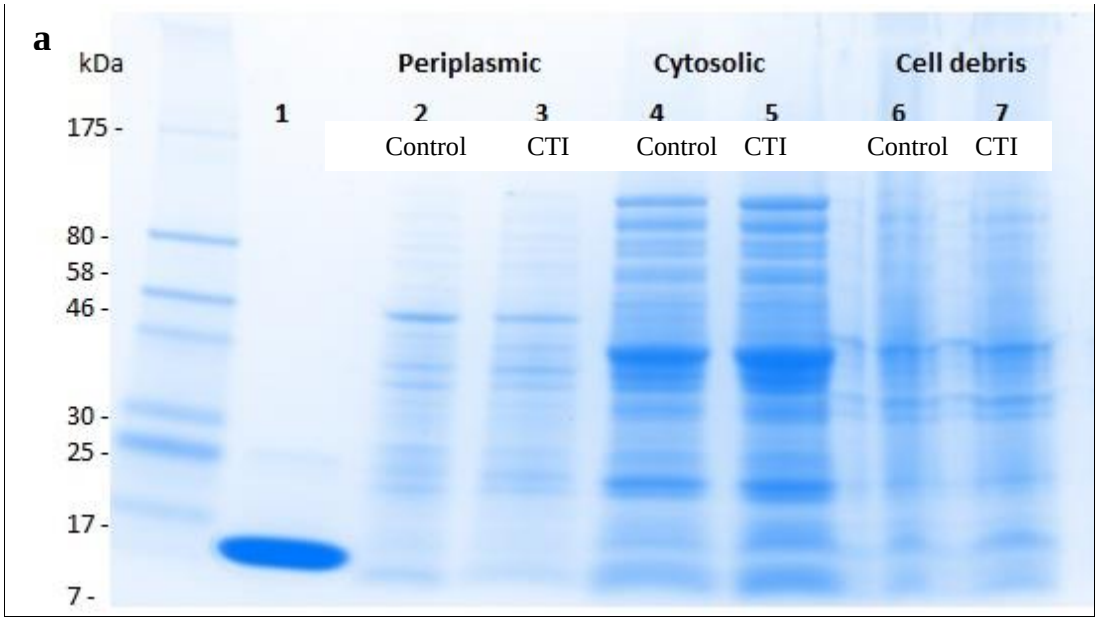
5.2.2 Expression of the *Cis-trans* isomerase (CTI) in *E. coli*

The *cti* gene from *Pseudomonas putida* DOT-T1E was codon optimised for expression in *E. coli* and was placed on a high copy plasmid pSB1C3 under the control of the *lac* promoter. In order to determine the ability of *E. coli* MG1655 to overexpress this heterologous gene, cells overexpressing the *cti* were grown in LB until they reached the early exponential phase and were induced with IPTG (90 µg/ml) for 4 hr. The periplasmic and cytosolic fractions of the cell were isolated as described in the Materials and Methods section (Chapter 2). The cell fractions of a control strain carrying an empty plasmid were also isolated. The different proteins present in each fraction were separated on an SDS-PAGE gel and was stained with

the Coomassie brilliant blue dye. Another gel ran in parallel with the same concentration of cell fractions was haem stained. The concentration of proteins run for both strains was normalised by loading ~8 µg and ~43 µg of periplasmic and cytosolic fractions respectively for both strains.

On the Coomassie-stained gel, the band pattern of the control strain was similar to that of the *cti*-overexpressing strain (Figure 5.1a). The CTI protein was not conspicuous on the gel suggesting that the amount loaded was too small to be detected by Coomassie staining.

Since haem proteins can be easily identified by determining their peroxidase activity when exposed to a chromogenic substrate such as TBMZ (Stugard *et al*., 1989), this method was used to detect the presence of the CTI. On the haem-stained gel, a band approximately 80 kDa was identified in the periplasmic and cytosolic fractions of the strain overexpressing the *cti* gene (Figure 5.1b) suggesting a successful overexpression of the gene. The lanes containing the cell fractions of the control strain were empty. There were no bands in the lanes containing the cell debris.



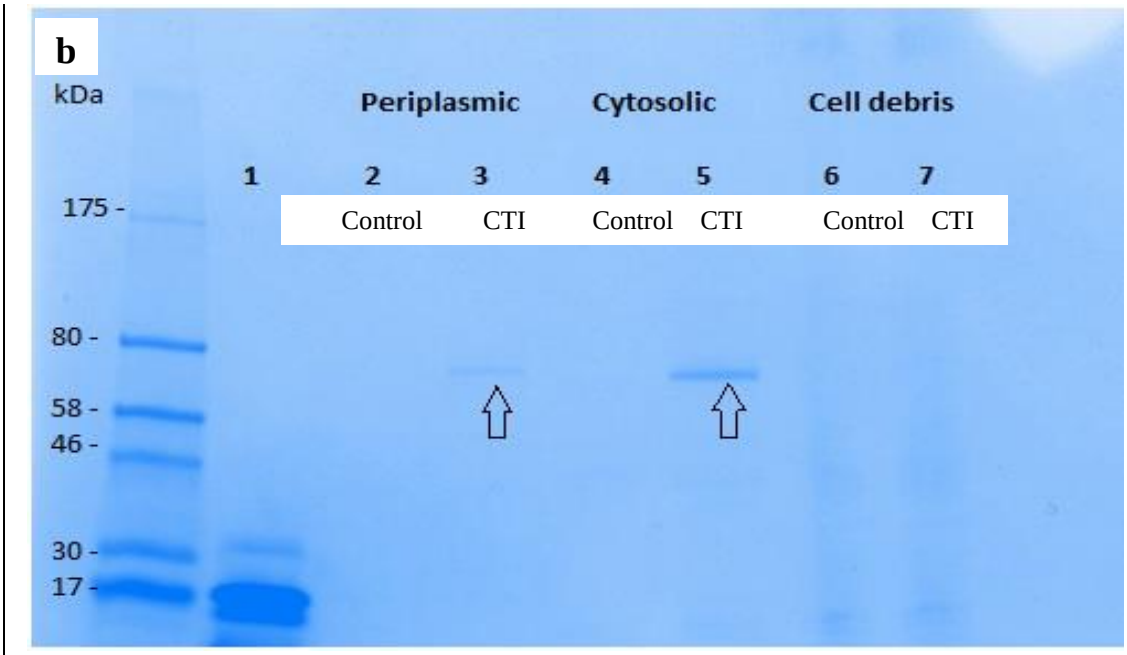


Figure 5.1: Expression of CTI in *E. coli* MG1655.

Proteins in the periplasmic fraction, cytosolic fraction and cell debris were ran on SDS-PAGE gels and stained with (a) Coomassie blue or (b) TMBZ. Lane 1 - pure cytochrome c; Lanes 2, 4, 6 – control strain and Lanes 3, 5, 7 – *cti* strain.

5.2.3 Effect of different levels of expression of the *cti* gene and temperature on *n*-butanol tolerance

In order to determine the effect of different expression levels of the *cti* gene on *n*-butanol tolerance *E. coli* MG1655 was transformed with the *cti* gene on either a high copy number plasmid, pSB1C3 or on a low copy number plasmid, pSB4K5. Time-course experiments were used to determine the effect of the *cti* overexpression on *n*-butanol tolerance. *E. coli* cells carrying this heterologous gene were grown in LB supplemented with increasing concentrations of *n*-butanol and were induced with IPTG (90 µg/ml).

Also, to test the hypothesis that *n*-butanol tolerance depends not only on *cti* overexpression but the temperatures at which the *cti* strain is grown, *E. coli* MG1655 overexpressing the *cti* gene was exposed to different concentrations of *n*-butanol at 30^{o}C or at 37^{o}C. Cells carrying the empty plasmids, pSB1C3 or pSB4K5 were used as control strains.

When exposed to low concentrations of *n*-butanol at 30^{o}C, all the different strains grew at a comparable rate (Figures 5.2 and 5.3). In 0.5% *n*-butanol, no difference in growth was observed in all four strains. The pSB1C3 control had a bizarre high growth in one of the experiments, hence the large standard error (Figure 5.3). However, at higher concentrations of the solvent as shown in Figures 5.5 and 5.6, cells overexpressing the *cti* gene grew better than their respective control strains even though those overexpressing the construct on pSB4K5 grew best. Even at a harsh concentration of 1.1% *n*-butanol strains overexpressing *cti* on pSB4K5 grew better than the other three strains. Growth of the *cti* strain in 1.1% *n*-butanol ended with an average final OD_{595} of 0.282 ± 0.008 which was significantly higher (p= 0.001) than

the pSB4K5 control whose growth stopped at an average OD_{595} of 0.186 ±0.001 (Figure 5.6).

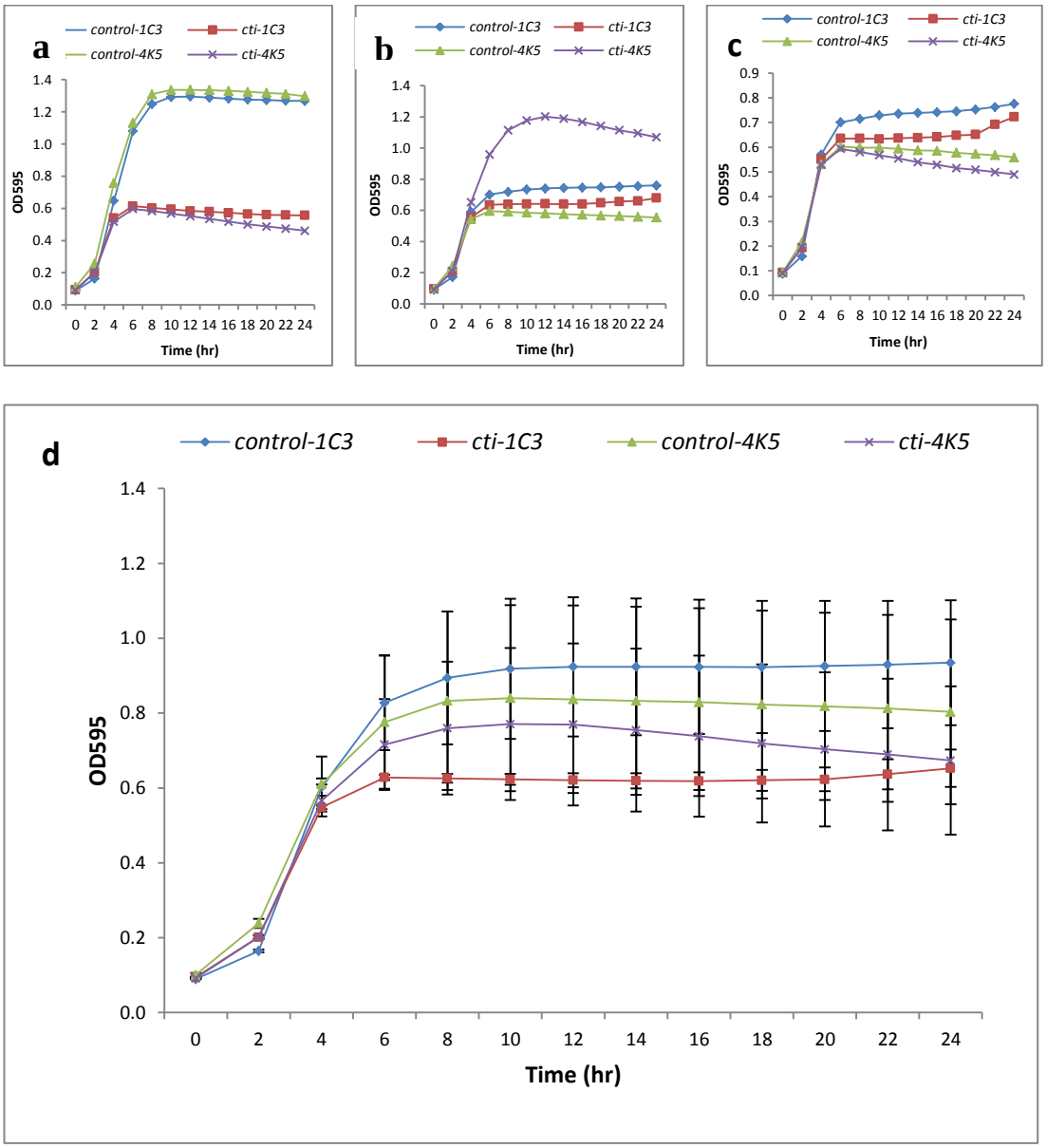


Figure 5.2: Effect of 0% *n*-butanol on the *cti* strain grown at 30°C.

A time-course experiment was conducted to measure growth of the *cti* and control strains under these conditions. The control strains carried an empty pSB1C3 or pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

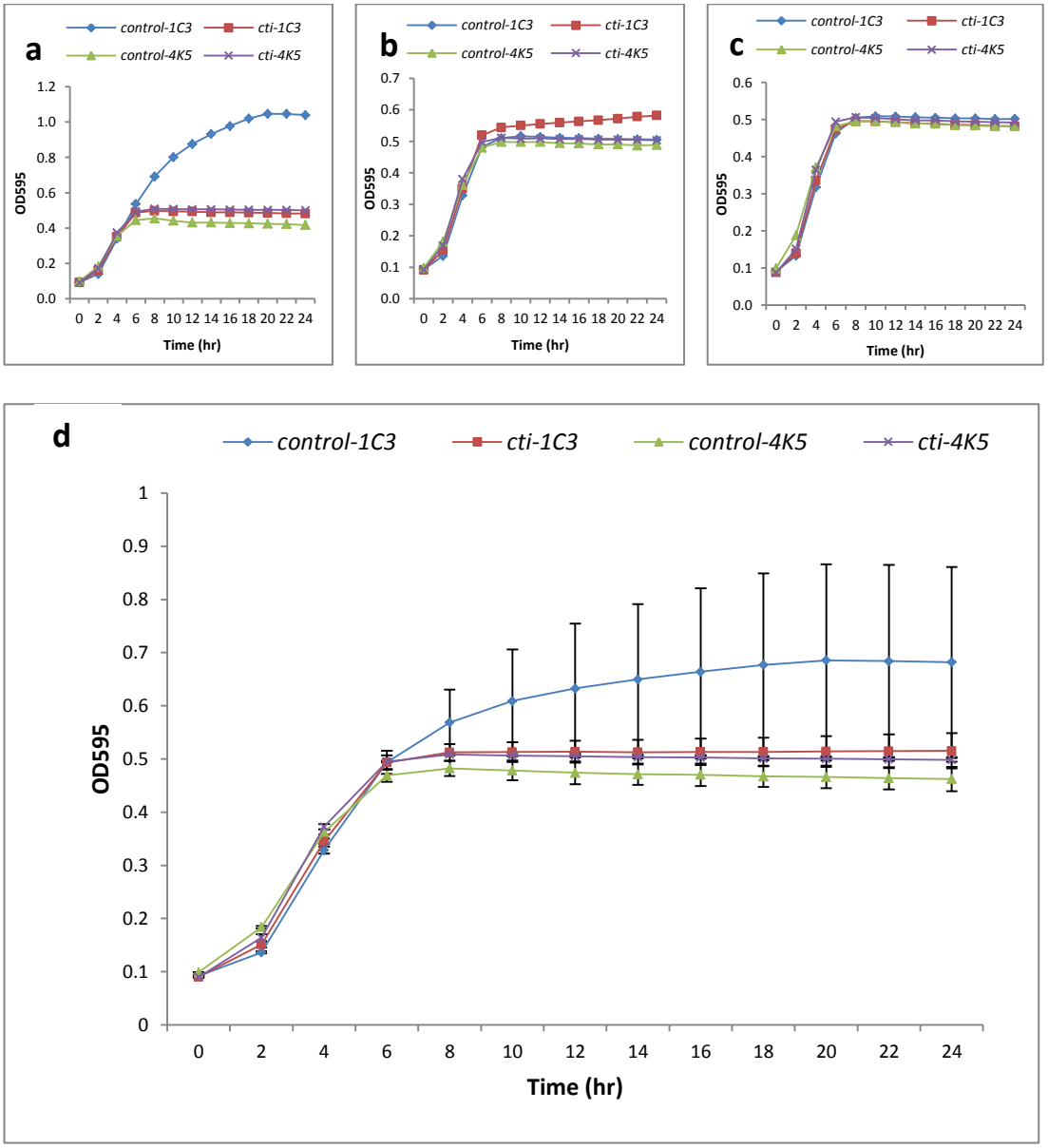


Figure 5.3: Effect of 0.5% *n*-butanol on the *cti* strain grown at 30°C.

A time-course experiment was conducted to measure growth of the *cti* and control strains under these conditions. The control strains carried an empty pSB1C3 or pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

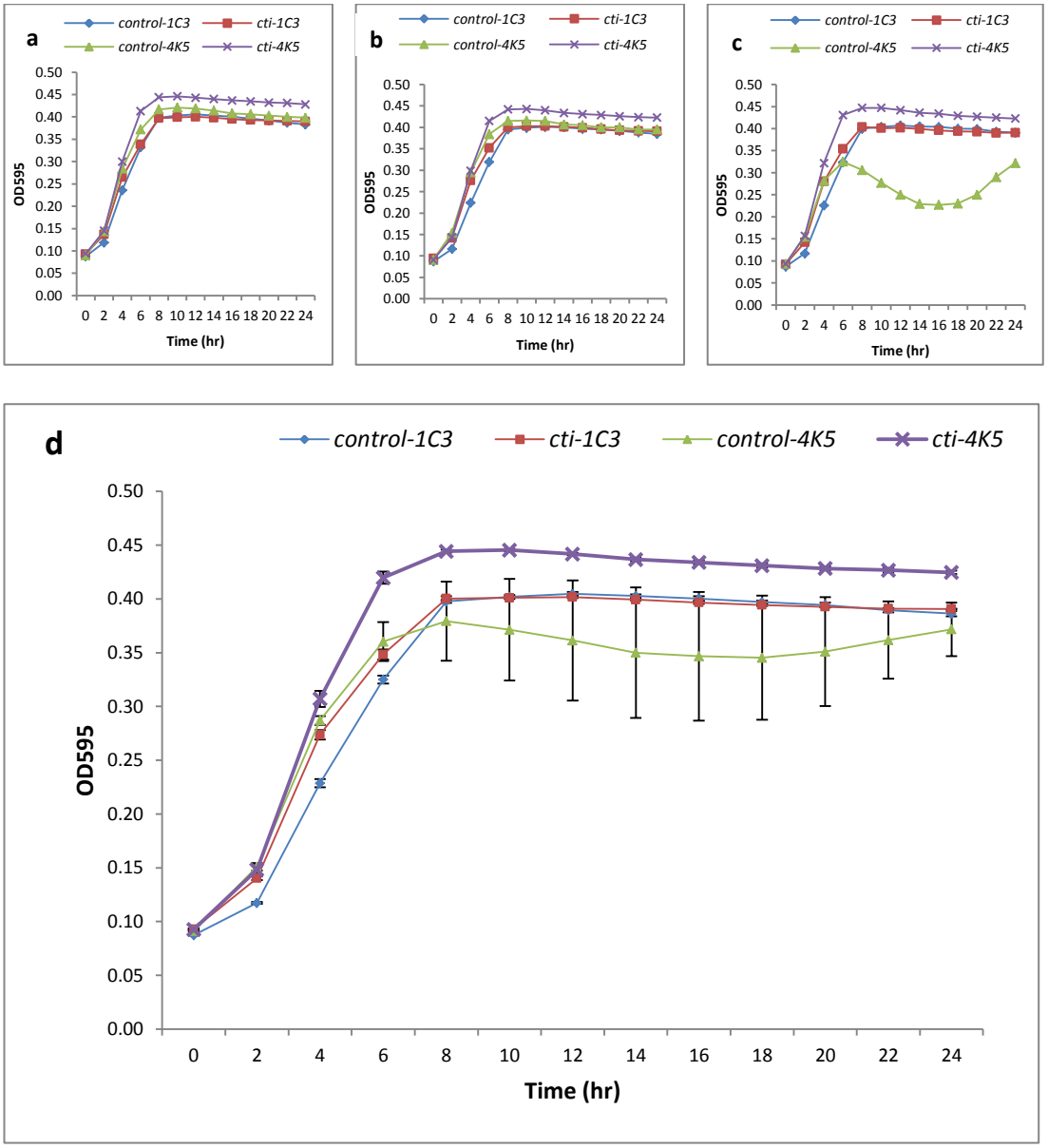


Figure 5.4: Effect of 0.7% *n*-butanol on the *cti* strain grown at 30°C.

A time-course experiment was conducted to measure growth of the *cti* and control strains under these conditions. The control strains carried an empty pSB1C3 or pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

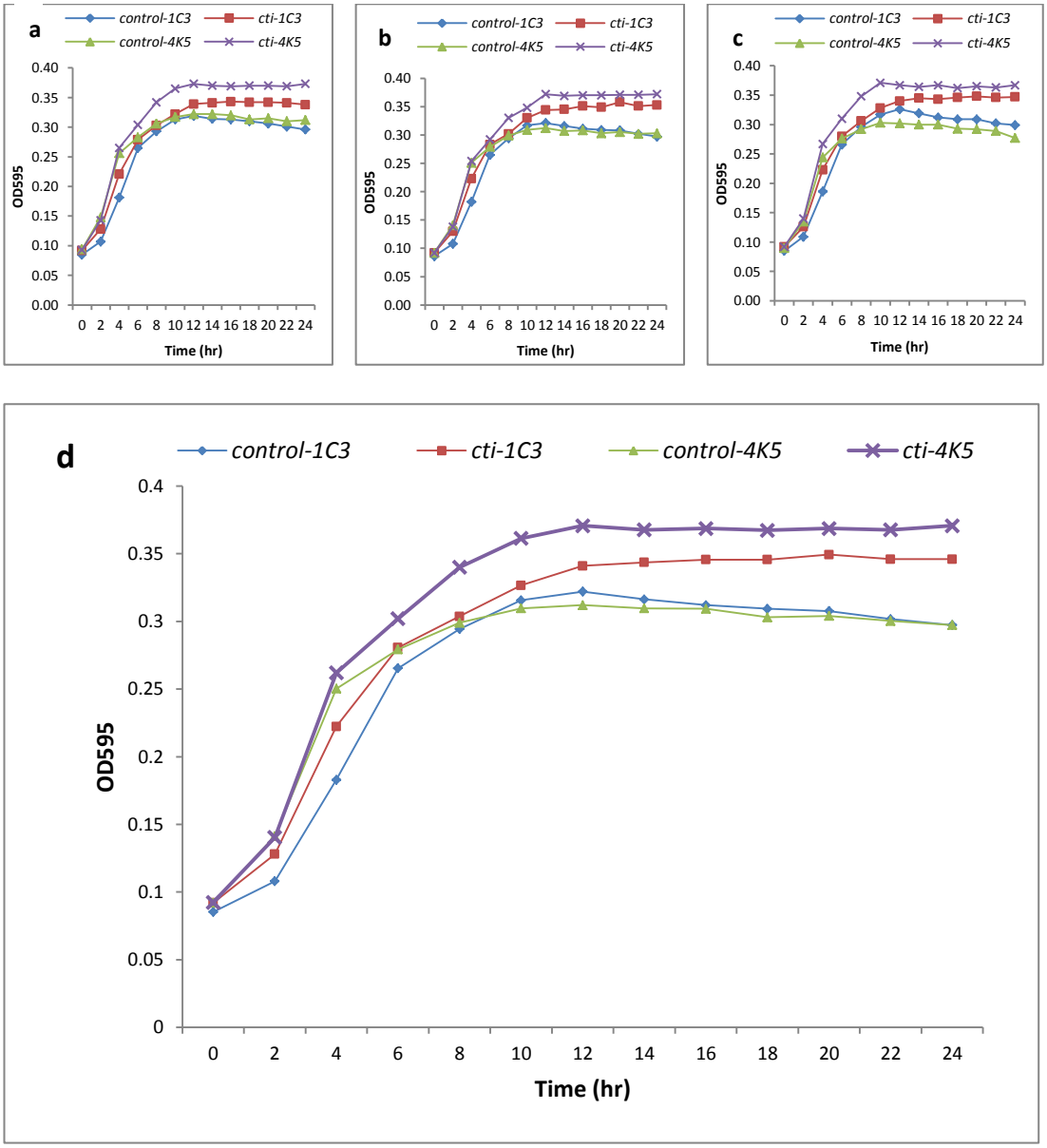


Figure 5.5: Effect of 0.9% *n*-butanol on the *cti* strain grown at 30°C.

A time-course experiment was conducted to measure growth of the *cti* and control strains under these conditions. The control strains carried an empty pSB1C3 or pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

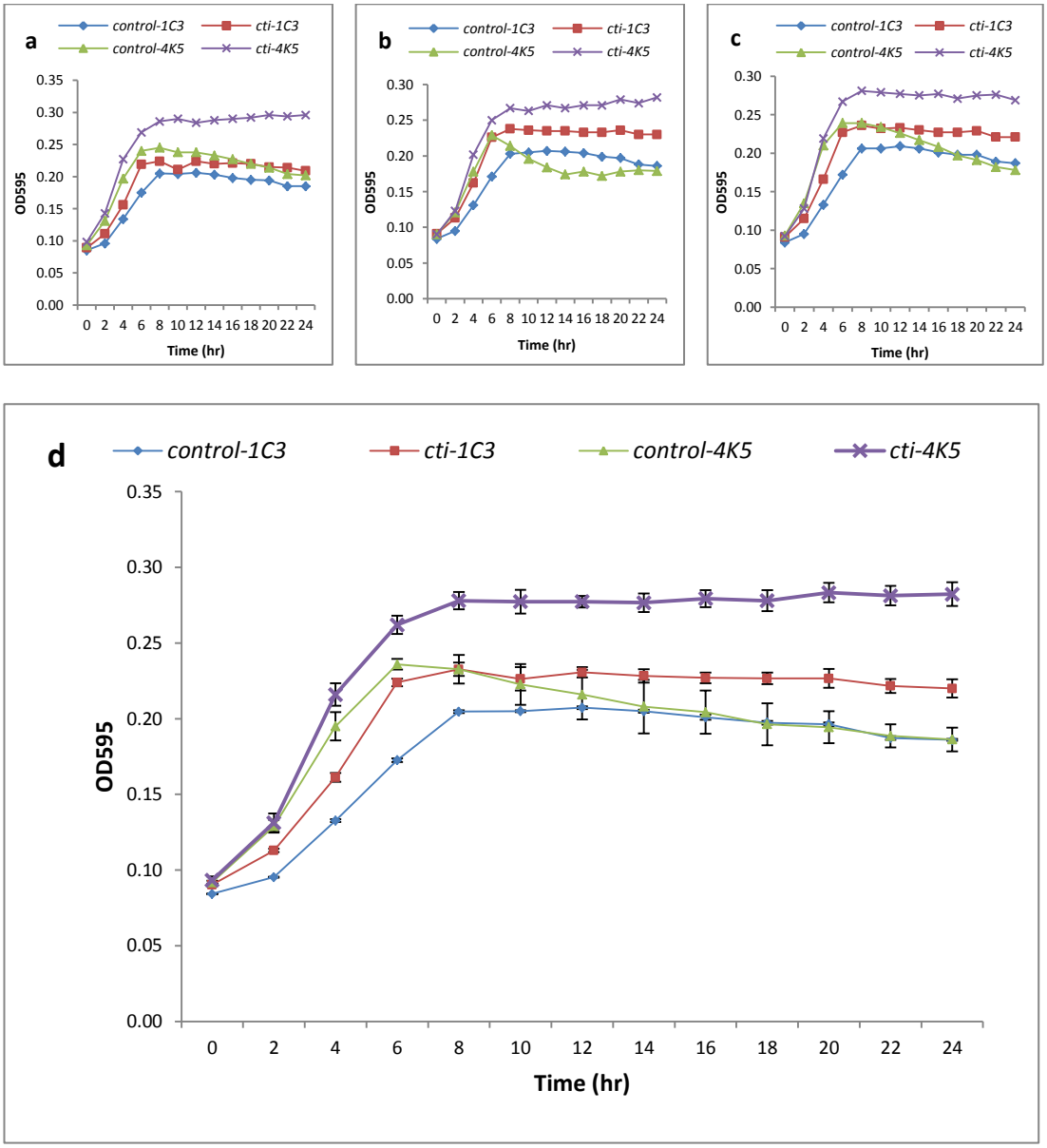


Figure 5.6: Effect of 1.1% *n*-butanol on the *cti* strain grown at 30°C.

A time-course experiment was conducted to measure growth of the *cti* and control strains under these conditions. The control strains carried an empty pSB1C3 or pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

At 37^{o}C, the different strains grew at a comparable rate in the absence of *n*-butanol. The control strain carrying an empty pSB1C3 appeared to grow better due to a peculiar OD_{595} reading in the first experiment which resulted in a huge standard error. In the other two experiments its growth curve was similar to the other three strains (Figure 5.7). When exposed to 0.5% *n*-butanol, strains overexpressing *cti* on pSB4K5 were more tolerant than the control strain carrying an empty pSB4K5. The inhibiting effect of the solvent on the control is more distinct (Figure 5.8). On the contrary, overexpressing the *cti* on pSB1C3 made the cells more sensitive to *n*-butanol compared to the control strain carrying the empty pSB1C3 plasmid. At higher concentrations the MG1655 strain overexpressing the *cti* on pSB4K5 were more tolerant than their control while those overexpressing the gene on pSB1C3 were more sensitive than their control (Figures 5.9 and 5.10).

Comparing the absorbance readings (OD_{595}) at the two different temperatures used in this experiment, the cells generally had higher OD_{595} readings when grown at 30^{o}C. Indeed, at a higher temperature, overexpressing the *cti* on pSB1C3 made the cells more sensitive to *n*-butanol relative to the control even though it increased tolerance to solvent at 30^{o}C. Strains overexpressing *cti* on pSB4K5 were more tolerant than the pSB4K5 control at both temperatures.

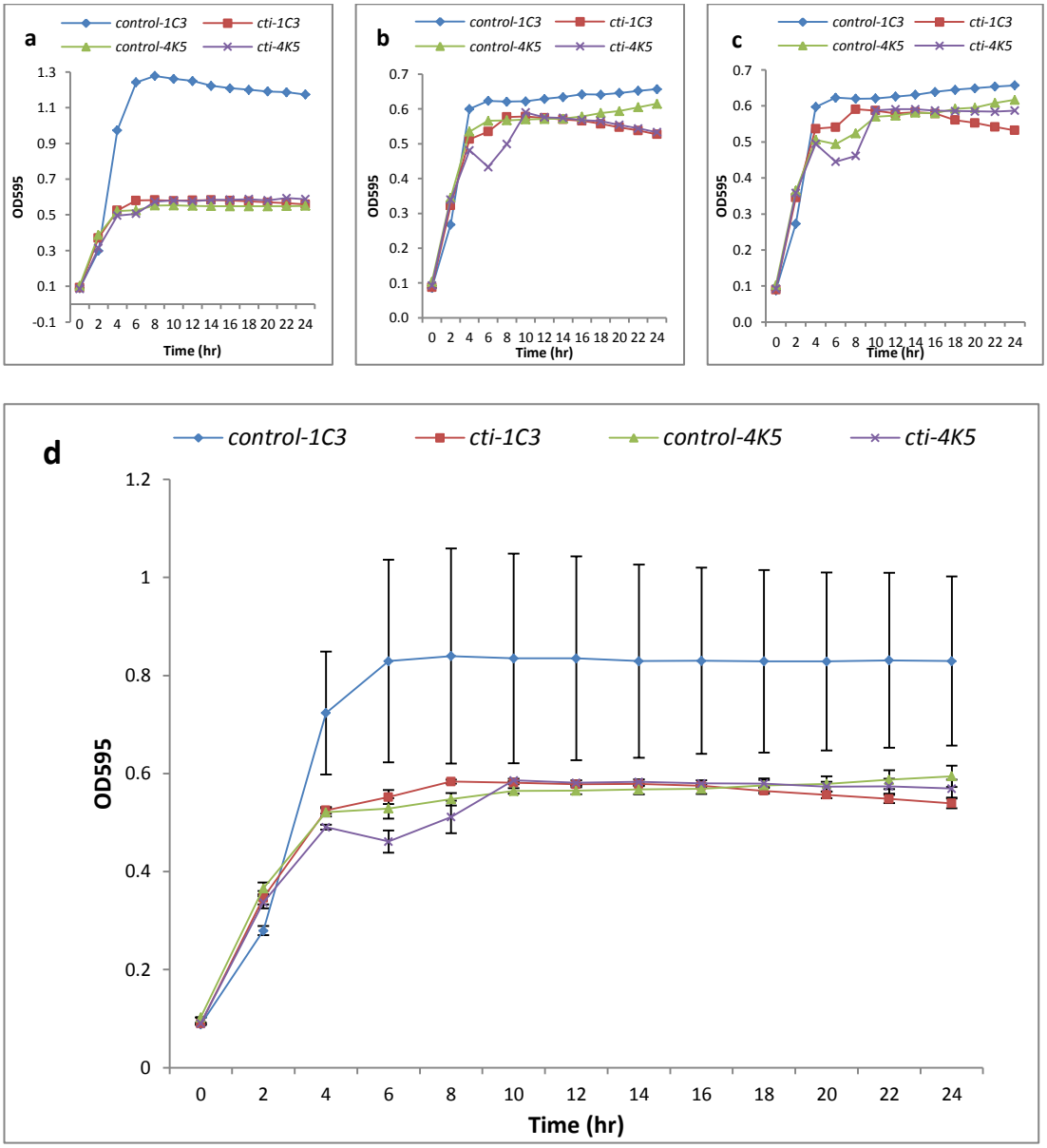


Figure 5.7: Effect of 0% *n*-butanol on the *cti* strain grown at 37°C.

A time-course experiment was conducted to measure growth of the *cti* and control strains under these conditions. The control strains carried an empty pSB1C3 or pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

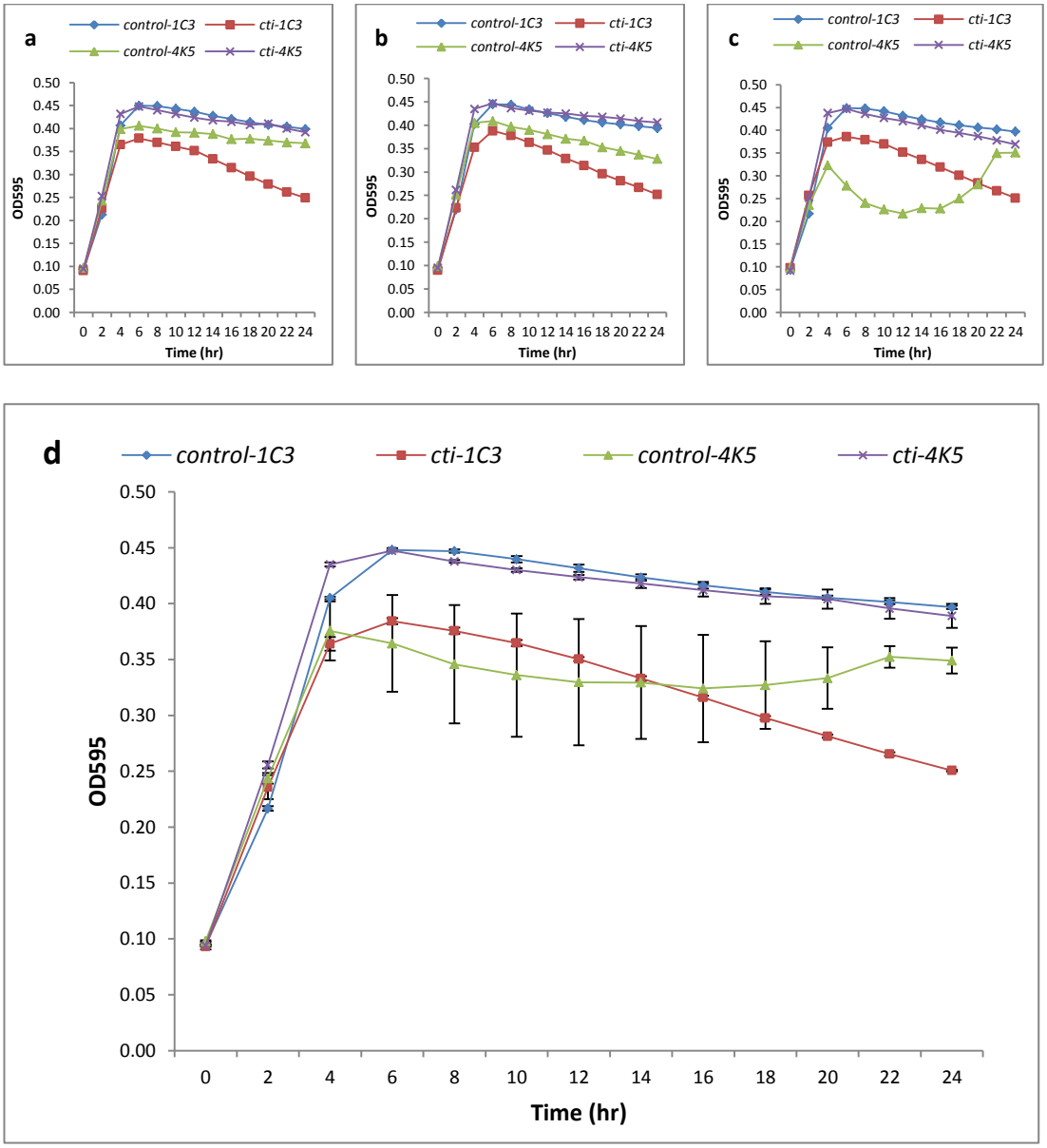


Figure 5.8: Effect of 0.5% *n*-butanol on the *cti* strain grown at 37°C.

A time-course experiment was conducted to measure growth of the *cti* and control strains under these conditions. The control strains carried an empty pSB1C3 or pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

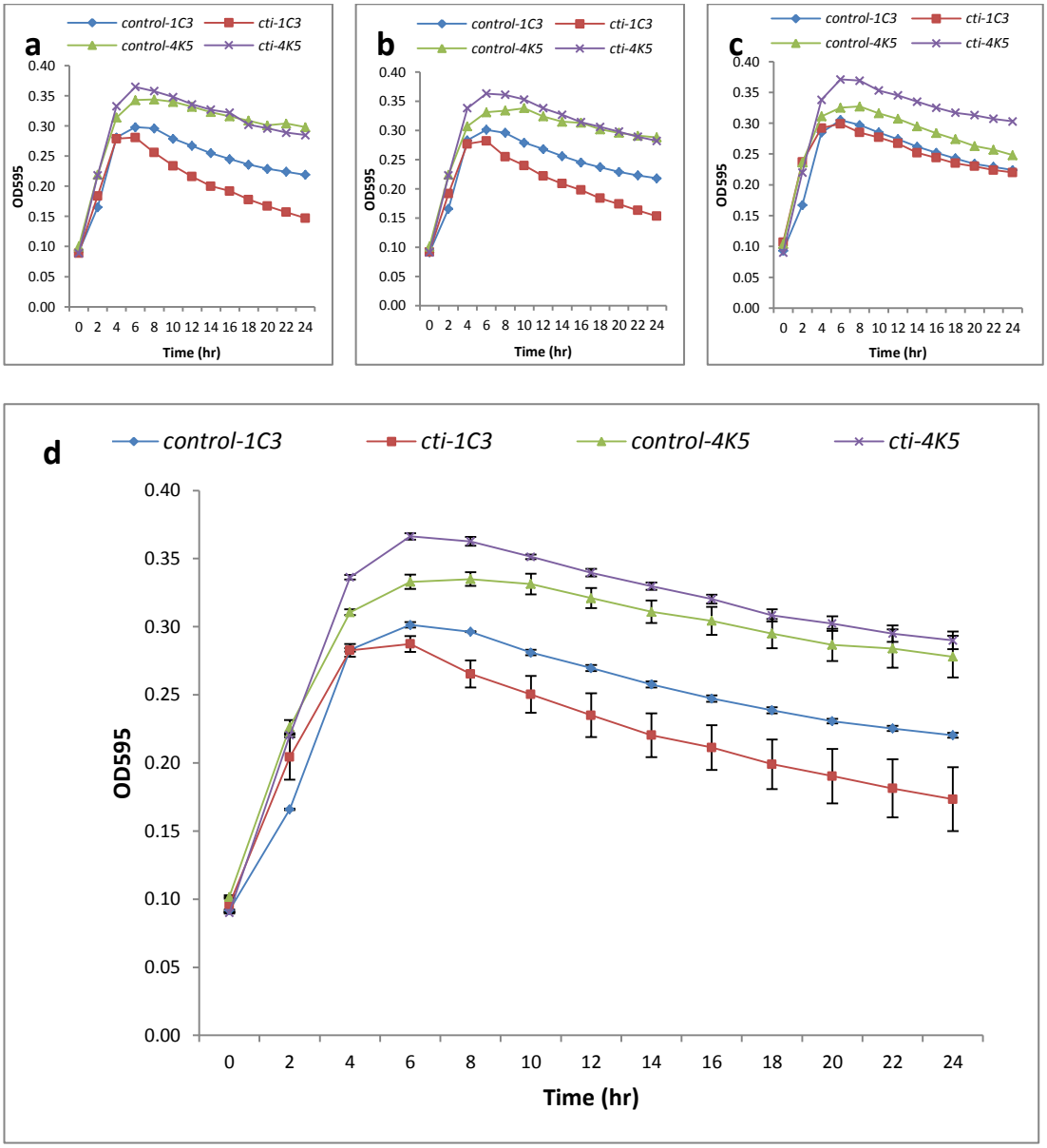


Figure 5.9: Effect of 0.7% *n*-butanol on the *cti* strain grown at 37°C.

A time-course experiment was conducted to measure growth of the *cti* and control strains under these conditions. The control strains carried an empty pSB1C3 or pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

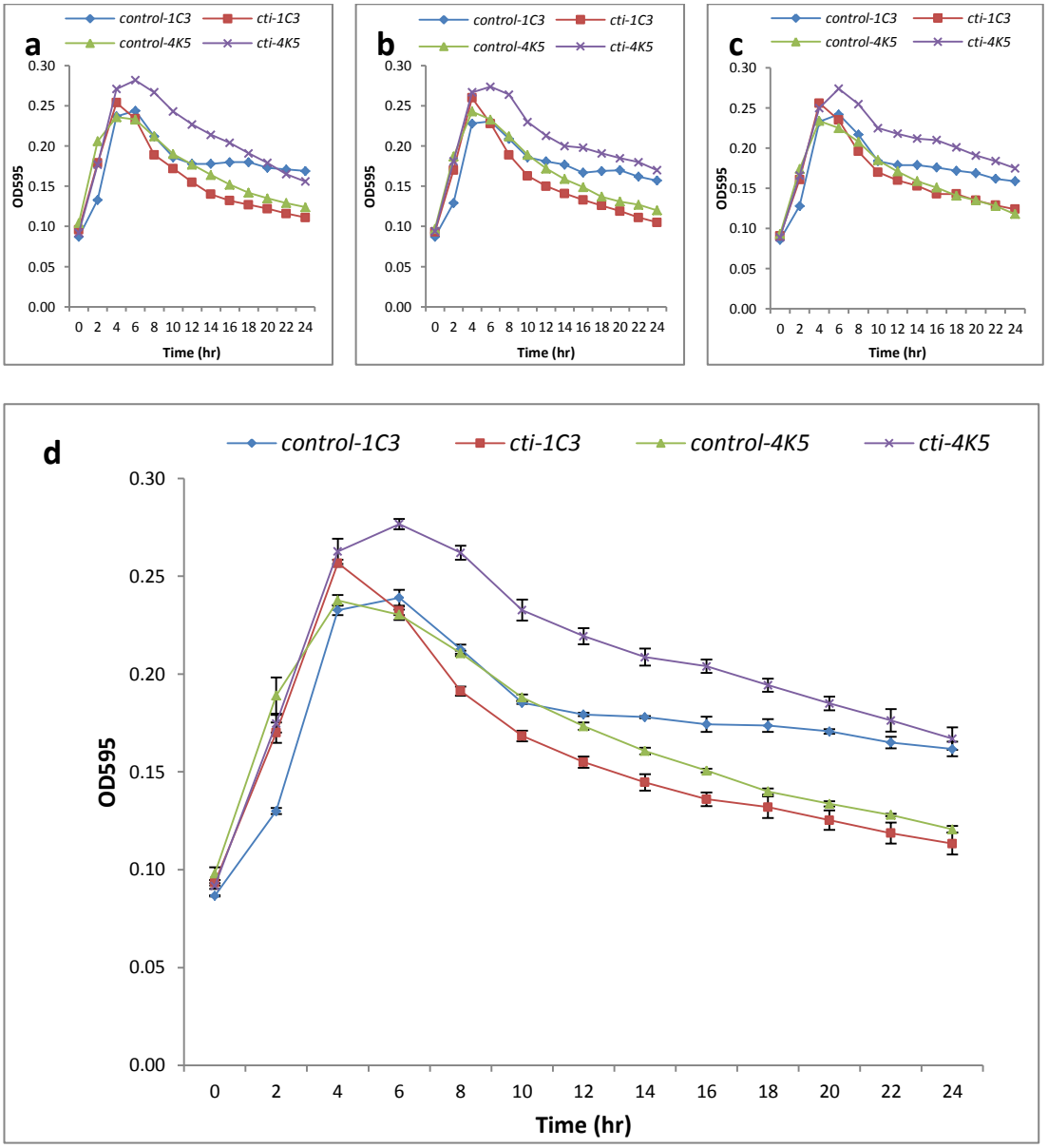


Figure 5.10: Effect of 0.9% *n*-butanol on the *cti* strain grown at 37°C.

A time-course experiment was conducted to measure growth of the *cti* and control strains under these conditions. The control strains carried an empty pSB1C3 or pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

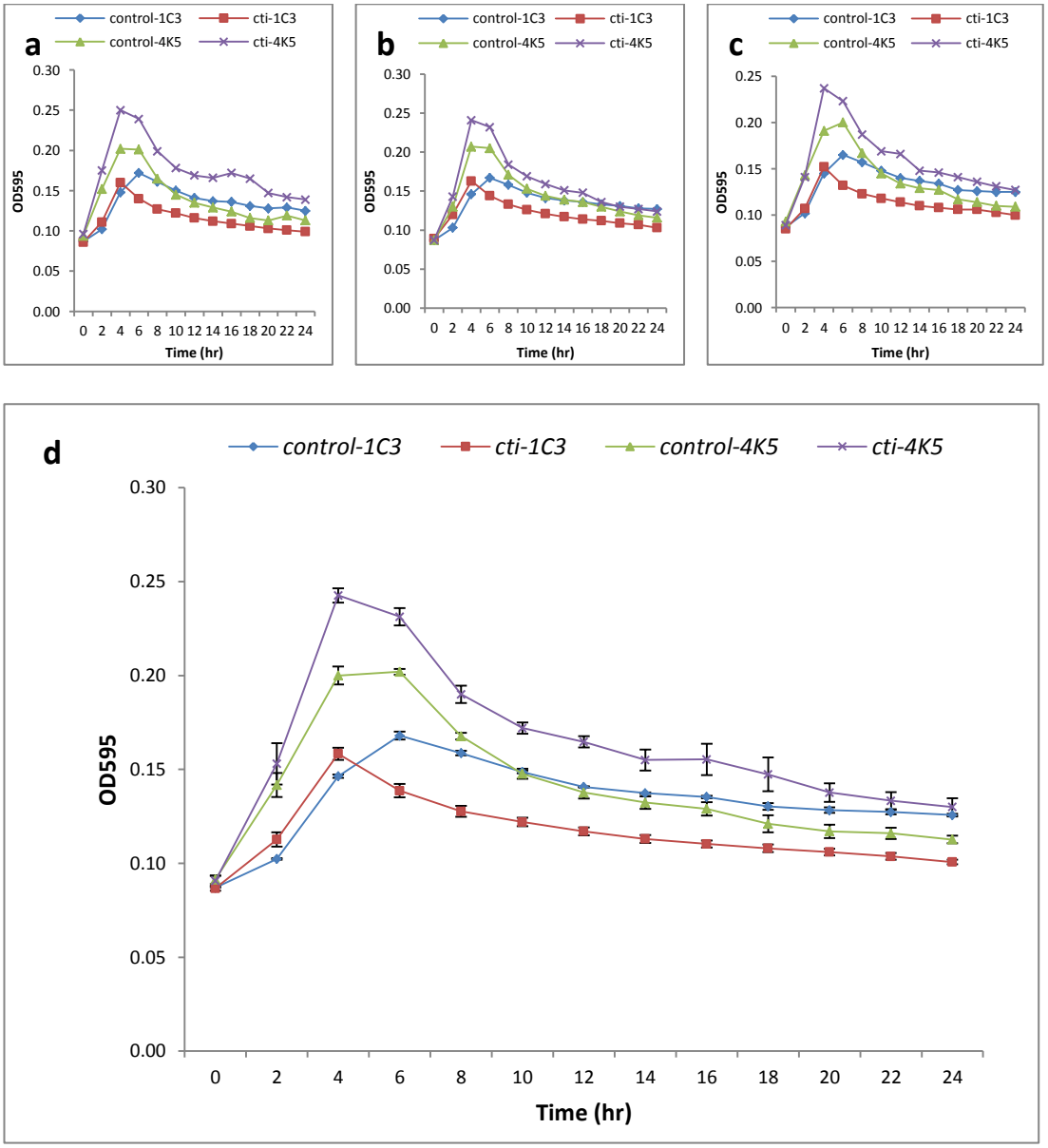


Figure 5.11: Effect of 1.1% *n*-butanol on the *cti* strain grown at 37°C.

A time-course experiment was conducted to measure growth of the *cti* and control strains under these conditions. The control strains carried an empty pSB1C3 or pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

5.2.4 *Cti* overexpression reduces membrane leakage

Based on the above observation that *cti* overexpression increased *n*-butanol tolerance. The hypothesis that this increase in tolerance is probably due to the protective effect of *cis-trans* isomerisation on the membrane which in effect, reduced membrane leakage in the presence of *n*-butanol was tested. In the previous chapter of this thesis (Chapter 4), the use of a fluorescence-based assay to detect membrane leakage was demonstrated. Therefore to determine the protective effect of *cti* expression on the membrane, *E. coli* MG1655 cells were co-transformed with the *cti* gene and *rfp* (DsRed) gene on two compatible plasmids. The *cti* was placed on pSB4K5 while the *rfp* gene was expressed on pSB1C3. A control strain was co-transformed with empty pSB1C3 and pSB4K5. The cells were grown in LB for ~4 hr at 30^{o}C till they reached the exponential phase after which *n*-butanol was added to the cultures to a final concentration of 1% v/v and further incubated at 30^{o}C. In a control experiment no *n*-butanol was added to the cultures. RFP leakage into the supernatant was detected by measuring fluorescence of the supernatants of the cultures at the start of the experiment and after 1 hr and 2 hr of incubation with *n*-butanol. The absorbance of the cultures at 600 nm was also measured and the specific fluorescence ratio was calculated as measure of membrane leakage since the amount of RFP produced is dependent on the number of cells in the culture.

At the start of the experiment, just before *n*-butanol was added to the cultures, the specific fluorescence ratio of the cultures of both control and *cti* strains was identical (Figure 5.12). However, after 1 hr incubation with the solvent, the specific fluorescence ratio of both strains increased with the ratio being significantly higher (p = 0.015) for the control cultures suggesting a higher level of membrane leakage compared to the *cti* strain. At 2 hr, the specific fluorescence ratio of both control and

cti strains further increased. There was no significant difference in the specific fluorescence ratio of both strains ($p = 0.095$).

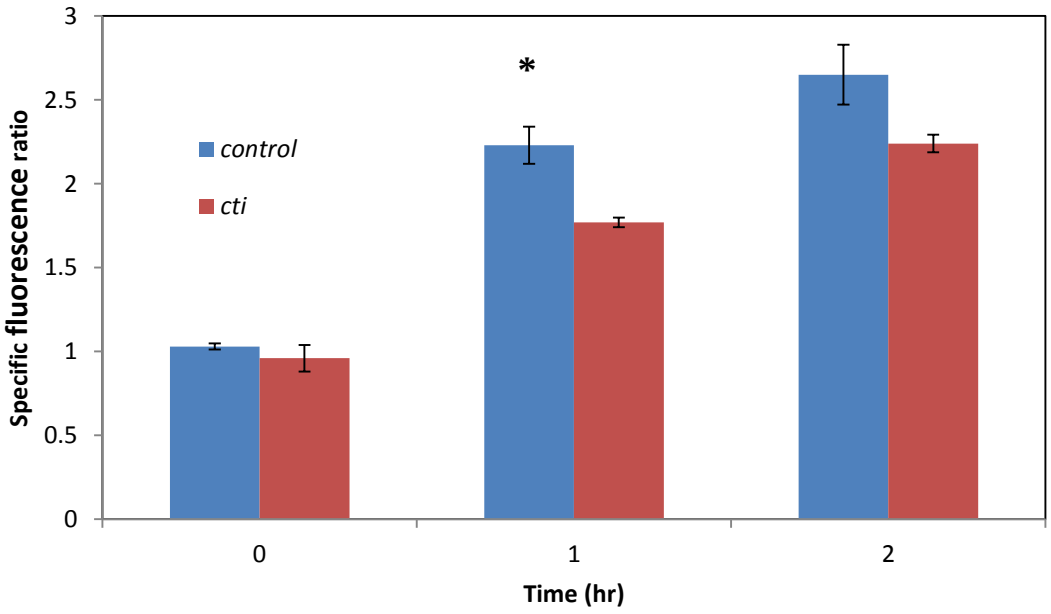


Figure 5.12: Effect of *cti* overexpression on membrane leakage.

E. coli MG1655 cells co-transformed with *cti* and RFP were challenged with *n*-butanol. RFP release into the supernatant was used to measure membrane leakage. Results presented show an average of three biological replicates. Error bars show standard error and asterisk represent significant difference ($p < 0.05$).

5.2.5 Morphological characterisation of the *cti* strain

To determine changes in the cell morphology after exposure to *n*-butanol, *E. coli* MG1655 was co-transformed with *cti* and *egfp* (enhanced GFP) on two compatible plasmids. The control strain carried *egfp* and an empty pSB4K5 plasmid. The cells were cultured in LB till they reached the early exponential phase. They were then further grown in LB supplemented with no *n*-butanol, mild (0.5%) and harsh (1.1%) concentrations of *n*-butanol. Changes to the cell shape were viewed under a fluorescent microscope before the solvent was added and after incubation for 1 hr. Before the solvent was added, the *cti* strain looked healthy and rod-shaped. However, after one hour of incubation, the cells became elongated (Figure 5.13). The cells exposed to 0.5% *n*-butanol maintained their original shape and did not look any different from those grown in the absence of the solvent. There were also fewer cells on the slide made from the 1% *n*-butanol cultures suggesting a reduction in cell growth.

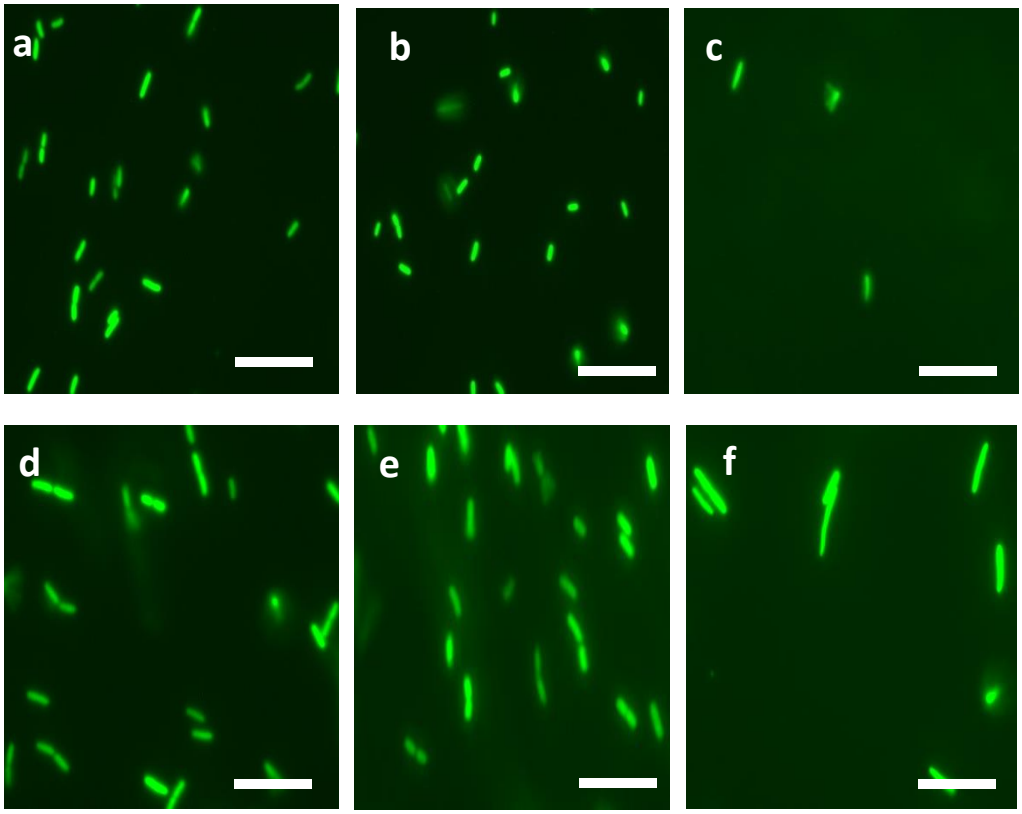


Figure 5.13: Effect of *n*-butanol on cell morphology.

Cells co-expressing the *cti* gene and EGFP were exposed to (a) 0% *n*-butanol, (b) 0.5% *n*-butanol and (c) 1% *n*-butanol and visualised with a fluorescent microscope. Control cells without the *cti* gene were also exposed to (d) 0% *n*-butanol, (e) 0.5% *n*-butanol and (f) 1% *n*-butanol. Scale bar represents 6 µm.

5.2.6 Determination of the effect of combining *cti* and *phPFD* on *n*-butanol tolerance

To test whether a combination of *cti* and *phPFD* (encoding a protein chaperone shown to significantly increase *n*-butanol tolerance in Chapter 3) will interact synergistically to further increase *n*-butanol tolerance, *E. coli* MG1655 was transformed with pSB4K5 carrying a combination of these genes under the control of the *lac* promoter which was induced with IPTG (90 µg/ml). Time-course experiments conducted to determine the protective effect of this combination on *E. coli* grown at 30^{o}C in LB supplemented with different concentrations of *n*-butanol.

In the absence of *n*-butanol, the *phPFD* and *cti* strains grew at a rate comparable to that of the control strain. The *cti+phPFD* strain, however, displayed a growth lower than the control suggesting that the overexpression of the gene combination posed some toxicity problems to the cell (Figure 5.14). When exposed to 0.5% *n*-butanol, the *phPFD* strain performed better the other strains (Figure 5.15). Consistent with earlier results presented in this chapter, the *cti* strain grows a rate comparable to the control strain at this concentration of *n*-butanol. At higher concentrations, *phPFD* still grows better than the other strains while the *cti* and *cti+phPFD* strains grow at a comparable rate even though they tolerate the solvent better than the control strain (Figures 5.15 and 5.16). The effect of the solvent increasing concentrations of *n*-butanol is more pronounced on the control strain than it is on the other strains. At a harsh concentration of *n*-butanol (1.1% v/v) growth of all the strains is drastically impaired. However, the *phPFD*, *cti* and *cti+phPFD* strains appear to tolerate that concentration of *n*-butanol better than the control strain with an average final OD_{595} of 0.232±0.001, 0.269±0.003 and 0.245±0.005 respectively while the control strain attains a final OD_{595} of 0.196±0.008 (Figure 5.17).

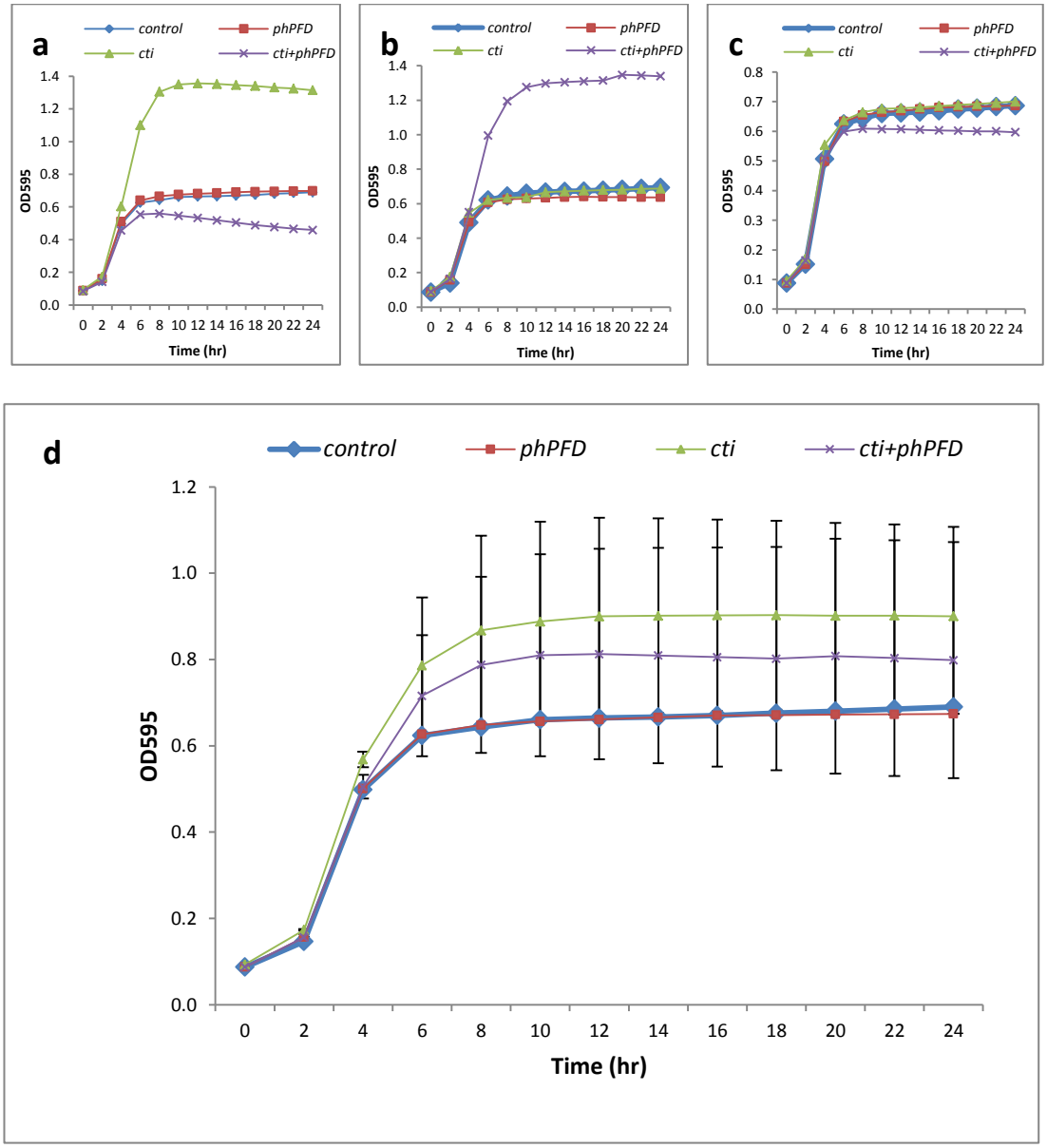


Figure 5.14: Time-course experiments of cultures without *n*-butanol.

The effect of *n*-butanol on the growth of the *cti*, *phPFD*, *cti+phPFD* and control strains are presented. The control strain carried an empty pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

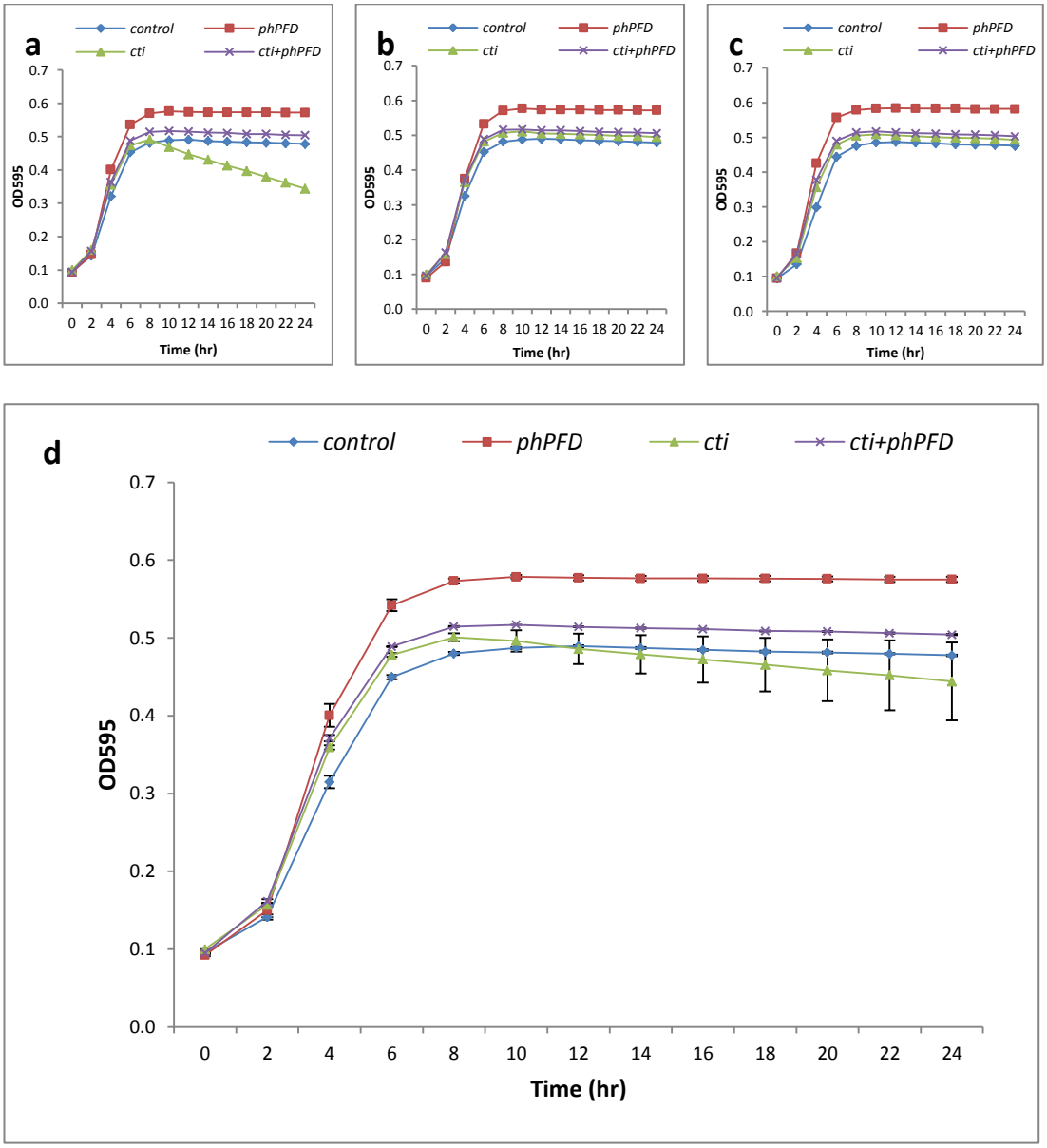


Figure 5.15: Time-course experiments of cultures containing 0.5% *n*-butanol.
The effect of *n*-butanol on the growth of the *cti*, *phPFD*, *cti+phPFD* and control strains are presented. The control strain carried an empty pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

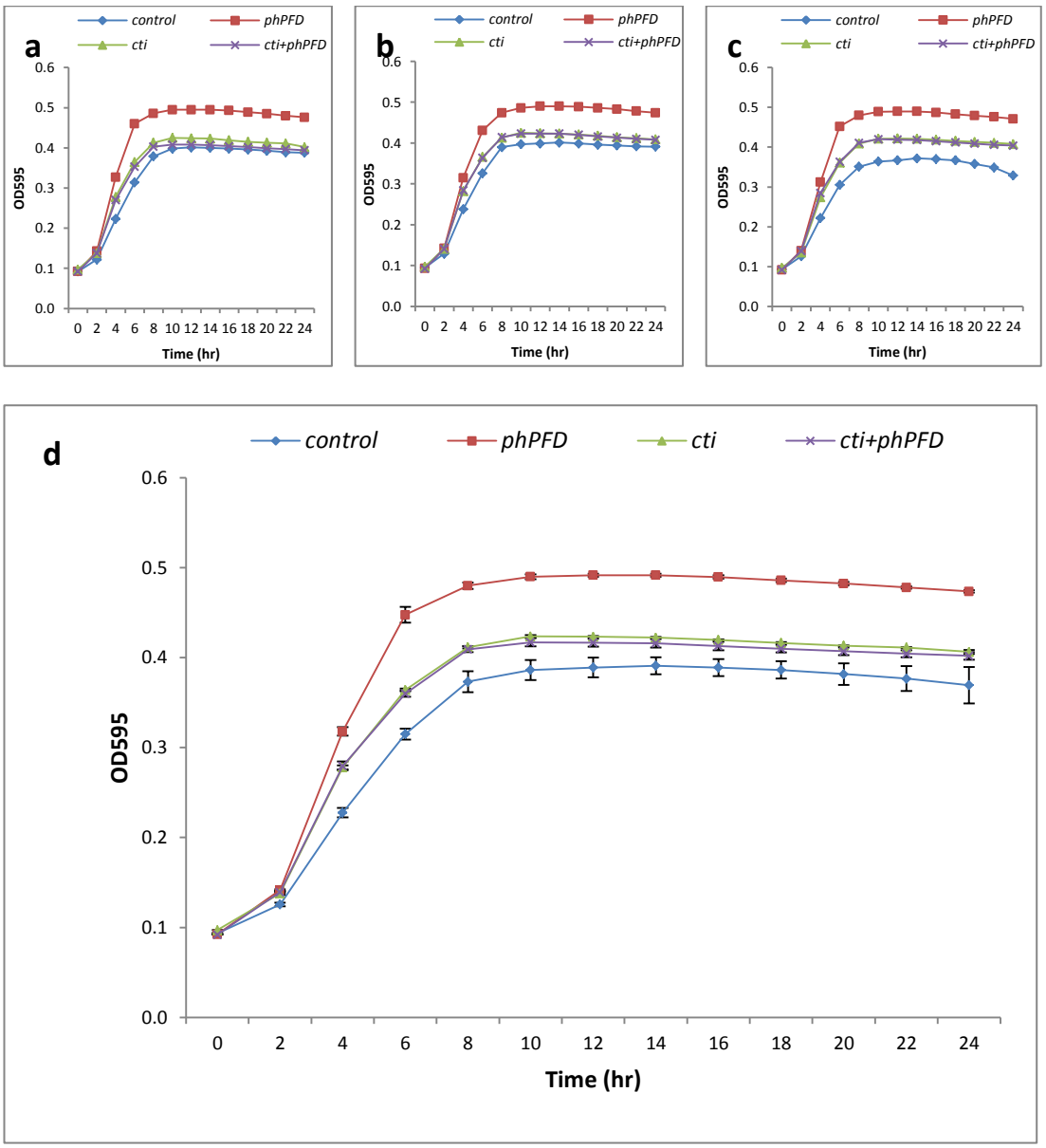


Figure 5.16: Time-course experiments of cultures containing 0.7% *n*-butanol. The effect of *n*-butanol on the growth of the *cti*, *phPFD*, *cti+phPFD* and control strains are presented. The control strain carried an empty pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

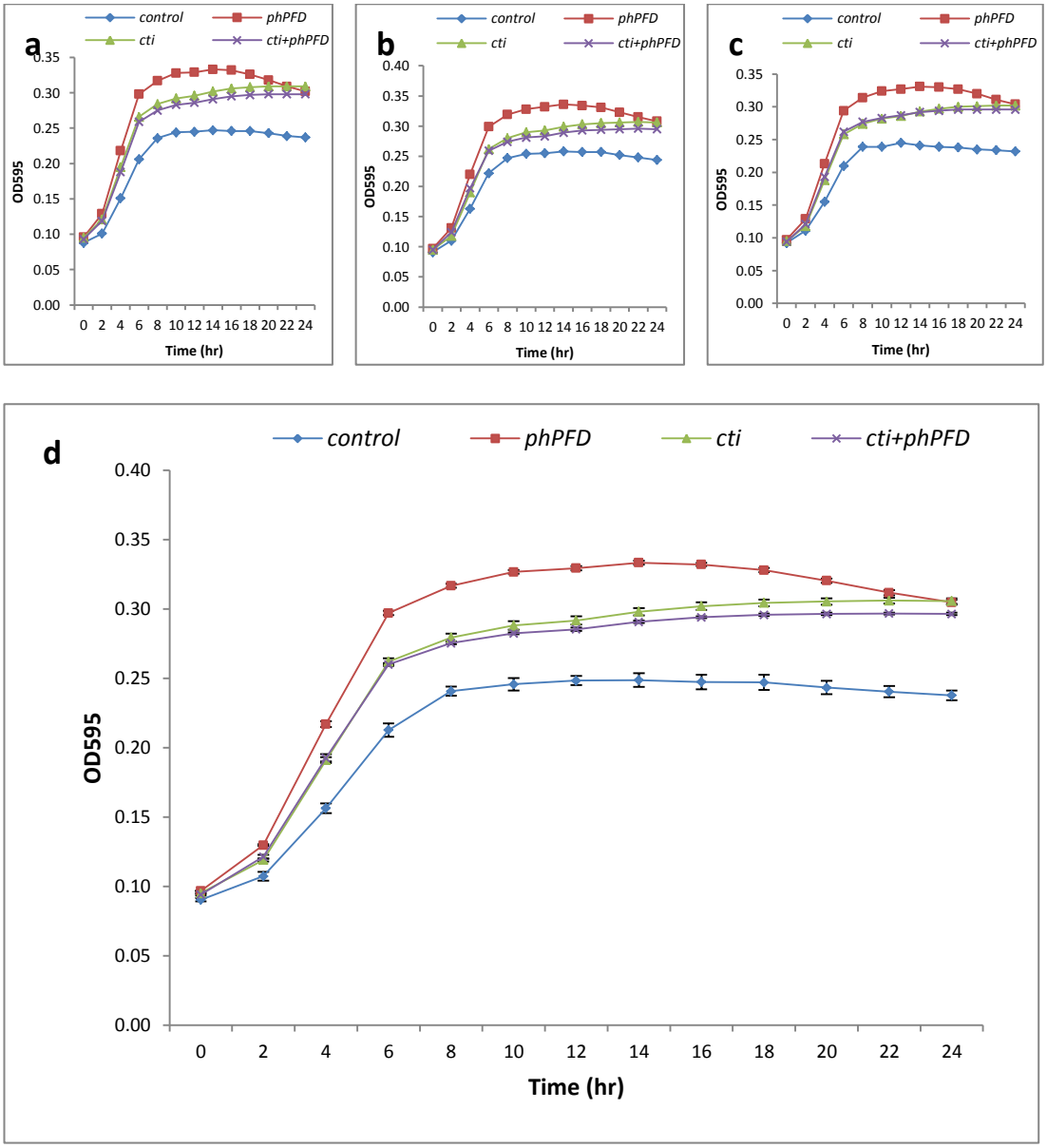


Figure 5.17: Time-course experiments of cultures containing 0.9% *n*-butanol.
The effect of *n*-butanol on the growth of the *cti*, *phPFD*, *cti+phPFD* and control strains are presented. The control strain carried an empty pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

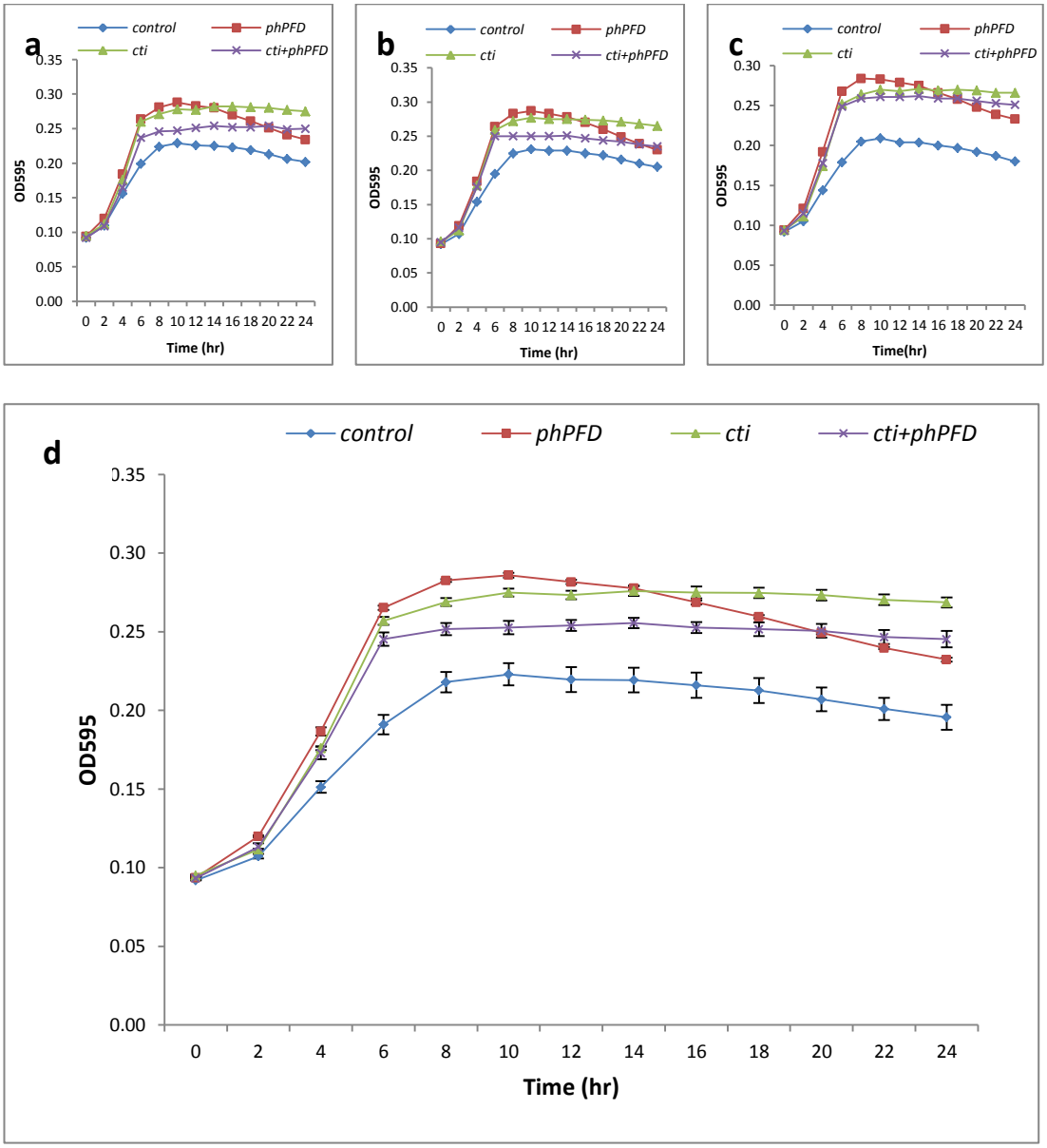


Figure 5.18: Time-course experiments of cultures containing 1.1% *n*-butanol.
The effect of *n*-butanol on the growth of the *cti*, *phPFD*, *cti+phPFD* and control strains are presented. The control strain carried an empty pSB4K5 plasmid. Graphs of individual experiments are shown in a, b and c. An average of the three is presented in d. Error bars indicate standard error of the three biological replicates.

5.3 Discussion

Regulating membrane fluidity is an important mechanism employed by some bacteria to resist harsh environmental conditions such as changes in temperature, osmotic shock and solvent stress. One key mechanism, among others, that *Pseudomonas putida* uses to counteract the effect of environmental stressors is reduction in membrane fluidity by converting the *cis* unsaturated fatty acids of membrane phospholipids into the *trans* form (Diefenbach *et al*., 1992).

In the present study, the possibility of increasing *n*-butanol tolerance in *E. coli* by the same mechanism of *cis-trans* isomerisation of unsaturated fatty acids in the cell's membrane was investigated. Even though the fatty acid *cis-trans* isomerase (CTI) which is responsible for this conversion is unique to *Pseudomonas* and absent in *E. coli*, a search through the Genbank database (http://www.ncbi.nlm.nih.gov/genbank/) was performed to identify any homologues of this protein in *E. coli*. A search through the NR (non-redundant) database failed to reveal any *E. coli* proteins homologous to CTI. The protein identified as the best hit was a liprotein, glycosyl hydrolase homologue which plays a role in degrading specific glycosides (Chhabra *et al*., 2002) and as such has no function identical to *cis-trans* isomerisation of fatty acids.

The *Pseudomonas putida* DOT-T1E *cti* gene was then codon optimised and successfully expressed and transported to the periplasm of *E. col*i MG1655 which was confirmed by haem staining. The isomerase when expressed in the cytoplasm is transported to the periplasm where a haem group is attached to it (Holtwick *et al.*, 1999). There were also some traces of the protein in the cytoplasmic fraction which is probably due to minor imperfections of the method used to isolate the periplasmic

fraction. In bacteria, since the attachment of haem to cytochrome c-type proteins occurs in the periplasm (Thony-Meyer *et al*., 2002), it should not be possible to observe a haem-stained protein in the cytosolic fraction of the cell. Also, the 80 kDa band identified on the gel corresponded to the size of a processed CTI consistent with a previous report by Pedrotta and Witholt, (1999).The successful expression and maturation of CTI in *E. coli* also means that the machinery involved in attaching a haem group to cytochrome c-type proteins in *E. coli* is also able to process heterologous proteins such as the CTI. This process is carried out by a number of proteins coded by the *ccmEFGH* operon in *E. coli* (Thony-Meyer *et al*., 1997) and there was no need to overexpress them or similar genes from *P. putida* in order to obtain the CTI in this study.

Since one of the effects of *n*-butanol on bacterial cells is leakage of cellular contents as a consequence of increase in membrane fluidity (previous chapter), engineering cells to counteract membrane leakiness is a step in the right direction to limit the effect of *n*-butanol on the membrane. However, expression of CTI at high levels on a high copy plasmid proved to be detrimental to the cell and made the cells rather more sensitive to *n*-butanol probably due to the load of the unfolded cytochrome c in the cytoplasm. These results agree with a previous report that tolerance to alcohols by bacteria is best achieved when its membrane fluidity is maintained and kept at constant (Huffer *et al*., 2011). Making the membrane too rigid creates further challenges to the cell and probably interferes with the transport of beneficial chemicals across it.

The effect of temperature and *cis-trans* isomerisation of the membrane on *n*-butanol tolerance was intriguing. The *cti* strain, in this study, grew better in the presence of

n-butanol at 30^{o}C than it did at a higher temperature of 37^{o}C. At 30^{o}C, the level of unsaturated fatty acids in bacterial membranes is higher (Heipieper *et al*., 1996; Holtwick *et al*., 1997) and this allows conversion of the *cis* isomer to the *trans* form. Furthermore, even though is CTI is constitutively expressed in *P. putida*, the *cis-trans* isomerisation is takes place when the cell encounters salts, organic solvents and changes in the temperature. The presence of organic solvents such as octanol have been shown to activate the CTI and initiate the *cis-trans* isomerisation (Holtwick *et al*., 1997; Pedrotta and Witholt, 1999) this might, therefore, explain why the *cti* strain was able to increase tolerance to *n*-butanol only at high concentrations of the alcohol in the present study.

When exposed to a relatively harsh concentration of *n*-butanol (1%), the *cti* strain was able to reduce RFP leakage suggesting that it fortified the membrane to an extent and prevented the solvent from damaging it. Exposure to the solvent, on the other hand, was also characterised by cell elongation and slowed growth suggesting that other mechanisms of toxicity were taking place intracellularly. Although inhibition of DNA synthesis has been reported to arrest cell growth resulting in filamentation of bacterial cells (Strauss *et al*., 2005), the direct effect of *n*-butanol on DNA damage and on key proteins involved in DNA synthesis has not been fully explored and requires further investigation. There is evidence, though, that genes involved in oxidative stress response are upregulated during *n*-butanol stress (Rutherford *et al*., 2010).

Although a combination of the *cti* and *phPFD* genes increased tolerance to *n*-butanol, there was no synergistic interaction between the two genes. Results obtained from this particular experiment also provided the opportunity to compare the

protective effect of a protein chaperone and a membrane modifying gene. It appeared from the present study that *phPFD* increased tolerance to *n*-butanol better than the *cti* and a combination of the two genes. This highlights the point that although membrane leakage occurs during *n*-butanol stress, damage to cytosolic proteins (not excluding membrane associated proteins) might be more prominent and that membrane leakage is one of the damaging effect of butanol on the cell but not the sole cause of butanol induced growth inhibition in bacteria (Huffer *et al*., 2011).

To conclude, results presented in this study show that *cis-trans* isomerisation of the membrane to reduce leakage of cellular contents provides another feasible option towards engineering solvent tolerant *E. coli*. Worth noting, however, is that although membrane leakage is detrimental to the cell, other factors such as maintaining the structure and function of cellular proteins should not be overlooked when developing tolerant strains. One would expect that a combination of membrane and protein protection during *n*-butanol stress would increase tolerance to the solvent. The inability to observe any synergistic interactions between these two mechanisms over-emphasises the need to explore novel methods to fully comprehend the complexity of the solvent tolerance phenotype. Future work should investigate how fold increase in the concentration of *trans* unsaturated fatty acids measured by gas chromatography corresponds with an increase in *n*-butanol tolerance.

Chapter 6: Discussion, Conclusions and Future work

Organic solvent tolerance in bacteria has been the focus of a lot of research in the past due to the potential benefits of using robust microbes in an industrial setting to produce economically important chemicals. Over the years, significant amount of work has gone into identifying naturally resistant microbes and developing tolerant strains by generating random mutations in the genomes of bacteria. More recently, using a rational approach to develop resistant bacterial hosts has been the highlight of work in this field. Applying novel molecular tools has enabled researchers to spot key genes that respond to solvent stress and to other stresses. A plethora of these genes have been listed in several publications (Goodarzi *et al*., 2010, Reyes *et al*., 2011; Rutherford *et al*., 2010). However, the role most of these genes play in solvent tolerance and the exact mechanisms of toxicity induced by organic solvents remains unclear. Also, it is important to test these genes individually to determine their effect on tolerance since the fact that a gene is upregulated during stress does not necessarily mean it increases tolerance to the stress. In this final chapter, the aims of this PhD project will be re-visited, put into perspective the significance or implications of key results obtained and make suggestions for future work.

In order to fill some gaps persisting in our knowledge of organic solvent tolerance, the aims of the present research were to identify genes (modules) suited for ethanol, *n*-butanol, furfural and acetone tolerance by employing different approaches to screen a library of stress response genes. Secondly, to determine the effect *n*-butanol

on the cell envelope of *E. coli* and finally, to increase *n*-butanol tolerance by reducing membrane leakage through *cis-trans* isomerisation of the membrane lipids.

Using three different methods including a bioluminescence assay, a library of stress response genes were screened for tolerance to ethanol, *n*-butanol, furfural and acetone. This bioluminescence screening, so far as I know, has not been used for this kind of screening before even though it has been used to detect environmental pollutants and other biocides on bacteria (Robinson *et al*., 2011). This screening provided an additional insight into the possible mechanisms of toxicity of ethanol and *n*-butanol. The top ethanol tolerance genes were *phPFD, marA* and *sodA* suggesting that the effect of ethanol on *E. coli* is prominent on the cell membrane and on cellular proteins. There is a likelihood of oxidative stress as well. It emerged that *n*-butanol also targeted the cell membrane and cytosolic proteins based on the top *n*-butanol tolerance genes which were identified as *phPFD*, *marA* and *glpC*. None of the genes in the library increased tolerance to furfural and acetone. It would have been ideal to assemble BioBrick parts into a detoxification pathway to deal with the furfural toxicity but this approach was beyond the scope of this research.

The existence of a heat shock response during ethanol and *n*-butanol stress cannot be over-emphasised due to the ability of a molecular chaperone from *Pyrococcus horikoshii*, Prefoldin, and the native *E. coli* heat chock response protein *groESL* to increase ethanol and *n*-butanol tolerance as shown in this research. Heat shock response is one of the major stress responses in living cells resulting in the induction of synthesis of heat shock proteins which mainly restore proteins damaged during stress (Rosen and Ron, 2003). Prefoldin from *P. horikoshii* has not been previously shown to increase *n*-butanol tolerance although its contribution to *n*-hexane tolerance

has been well established (Okochi *et al*., 2007). Overexpression of the *E. coli* molecular chaperone, *groESL* resulted in increased tolerance (Zingaro *et al*., 2012) consistent with my results. Therefore, based on these observations, heat shock response should be incorporated into rational design of tolerant microbes.

Protein denaturation and restoration should also be further investigated. Future work should investigate what proteins are targeted by *n*-butanol. It will be interesting to identify specific processes and their associated proteins whose functions are gravely affected by *n*-butanol especially in *n*-butanol producing strains where the solvent is produces intracellularly. An example of one such biological process which can be impaired by ethanol and *n*-butanol is DNA synthesis. Maintaining the activity of enzymes involved in DNA synthesis is important for cell growth and division. Investigating the effect of *n*-butanol and other organic solvents on such enzymes will be beneficial in understanding the how these chemicals inhibit growth.

The involvement of a transcriptional activator, *marA*, in ethanol and *n*-butanol tolerance was quite interesting. This gene controls the expression of several other genes including the *acrAB-tolC* system used by bacteria to export antibiotics out of the cell (Martin *et al*., 1997). Similarly, the efflux of ethanol and *n*-butanol out of the cell *via* this mechanism might explain the role of *marA* in alcohol tolerance. Conflicting results existing in literature about the role of *marA* in ethanol tolerance was indirectly addressed in this project. In the present work, *marA* was found to increase tolerance to both ethanol and *n*-butanol and these results were also confirmed by time-course experiments. Contrary to findings presented here and similar work done by others (Chong *et al*., 2013), it has been reported that *marA* does not increase tolerance to simple alcohols such as ethanol and 1-propanol (Ankarloo

et al., 2010). Similarly, *marAB* overexpression has been mentioned as not having any effect on ethanol tolerance (Gonzalez *et al*., 2003). The inability of Ankarloo *et al*. (2010) to observe ethanol tolerance when *marA* was overexpressed was probably due to the approach they used to investigate ethanol tolerance. In that paper, cells were challenged with a single concentration of ethanol (10% w/w) which is high enough to inhibit the most ethanol tolerant *E. coli* strain. Furthermore, it emerged from my work that when the *marA*-overexpressing strain was exposed to ethanol, it appeared sensitive to the alcohol during the first few hours of exposure and then recovered to outgrow the control strain. Therefore the time at which tolerance is measured plays an essential role in getting a clearer understanding of phenotype. This is another reason why ethanol tolerance was not observed by Ankarloo *et al*., (2010) since they determined growth in the presence of ethanol after 30, 60 and 90 min of incubation. This emphasises the importance of assessing tolerance at the different phases of bacterial growth and the need to employ multiple assays to show the effect of the organic solvents at different growth phases of bacteria. The exact reason for the long lag phase of *marA*-overexpressing strains should be further explored. The use of a more standardised approach to explore solvent toxicity will also help eliminate inconsistencies in results from different research groups.

The bioluminescence assays used in this work played an essential role in complementing the traditional methods used to assay for solvent tolerance. The major issue that restricts its application is the fact that it depends on the metabolic activity of the cell. Therefore, when cells are in a low metabolic state such as during the stationary phase, its application becomes limited. However, it still remains a useful tool for characterising the effect of toxic chemicals on the cells during their exponential phase of growth. The assay should be further developed to allow high-

throughput screening of a wider variety of other genes or mechanisms involved in solvent tolerance against a broader range of industrially relevant chemicals.

The results obtained in Chapter three were followed up on by studying the actual effect of *n*-butanol on membrane integrity and cell shape. A fluorescence-based method was developed to achieve this objective. Using this assay and the alkaline phosphatase assay, it was possible to quantitatively detect leakage of the inner and outer membranes of *E. coli* respectively. This provides an alternative and more reliable way of assaying membrane leakage unlike previous reports that measured membrane leakage by assaying for the presence of extracellular amounts of magnesium ions (Royce *et al*., 2013; Zaldivar *et al*., 1999). Magnesium ions are essential for growth of bacteria and as such, are found in most growth media. Depending on the media used to grow the cells for the magnesium leakage assay, it will be difficult to interpret results due to presence of some amounts of the ions already present in the growth media.

Further work was done to investigate the effect of *n*-butanol on the outer membrane using the *lptA* bioreporter and the purpald assay. The *lptA* promoter which is induced during outer membrane damage was responsive to *n*-butanol. *n*-Butanol was also found to remove LPS molecules from the outer membrane of *E. coli*. This led me to believe that *n*-butanol is able to get into the cell by breaking the resistance created by the hydrophilic LPS. Once this barrier is broken, the solvent intercalates into the phospholipid bilayer and eventually enters the cells. Intercalating into the phospholipid bilayer renders the membrane leaky as result of increased membrane fluidity. This proposition explaining the possible mechanism by which *n*-butanol enters the cell has not been previously reported, to the best of my knowledge. It

provides an opportunity for rational engineering of microbes from another perspective by modifying and reinforcing the hydrophilic LPS barrier on the membrane to prevent *n*-butanol from entering the cell. The possibility of *n*-butanol entering cells *via* porins on the outer membrane should not be overlooked. Porin mutants should be tested for *n*-butanol accumulation in the cell and then screened for butanol tolerance in order to identify the role porins play in transporting *n*-butanol across the membrane.

E. coli cells became elongated when exposed to *n*-butanol concentrations higher than their maximum tolerable concentration suggesting an element of inhibition of DNA synthesis or damage to DNA probably due to oxidative stress. However, over-expression of some oxidative stress response genes in Chapter three failed to increase tolerance to *n*-butanol. Indeed, in some cases, *sodA* overexpression made the cells even more sensitive to the solvent despite results from transcriptional studies showing it is highly upregulated during *n*-butanol stress. Overexpression of *sodA* alone might be detrimental to cells due to the accumulation of hydrogen peroxide. Therefore its enrichment in transcriptional studies imply that it may be working with other oxidative stress response genes to eliminate the toxic effect of butanol possibly by detoxifying potential reactive oxygen species produced by the solvent. This comes to confirm the fact that upregulated genes do not necessarily increase tolerance especially when it is isolated from the network of genes responsible for increasing tolerance.

The possibility of increasing *n*-butanol tolerance by modifying the membrane of *E. coli* with a *Cis-trans* isomerase (CTI) from *Pseudomonas putida* was investigated in Chapter six. Tolerance to *n*-butanol tolerance was increased when the isomerase was

expressed at low levels and when the cells were cultured at 30 ^{o}C instead of 37 ^{o}C consistent with a previous report suggesting that CTI activity is optimal at 30 ^{o}C (Pedrotta and Witholt, 1999). An attempt to further increase tolerance by combining the *cti* gene with the molecular chaperone gene, *phPFD* proved futile even though the combination was able to increase tolerance to *n*-butanol. Results from this experiment also showed that *phPFD* when overexpressed individually increased *n*-butanol tolerance better that the *cti* gene when overexpressed alone re-emphasising the point that membrane damage, though serious, is not the main cause of *n*-butanol-induced cell death.

The major challenge faced in this PhD research was the inability to further increase solvent tolerance by combining different tolerance genes. This re-emphasises the complex nature of *n*-butanol tolerance. The problem was not resolved when the gene combinations were expressed at a lower level and induced with different concentrations IPTG. Further work should go into optimising expression of the individual genes when several genes are combined. Again, more experiments should be carried out to measure the expression levels of each of the genes combined by using RT-PCR, for example, as co-expression probably generates lower levels of expression of the individual genes. By using a multiplasmid system, it might be possible to directly control the expression levels of the individual genes (Zingaro *et al*., 2012). The downside to using multiple plasmids is that cell growth is compromised due to the extra metabolic burden placed on the cell to replicate the plasmid and the multiple plasmids will induce an oxidative stress which could be detrimental to the cells (Silva *et al*., 2012). The effect of different promoters and ribosome binding sites on expression of gene combinations can also be explored. Another approach will be to insert the gene combination into the genome of the

bacterial host. New directions in synthetic biology have focussed on engineering whole genomes and this has resulted in recent efforts to develop tools that can make genome editing possible (Enyeart *et al*., 2013; Hughes and Maloy, 2007). Such strategies could be used to insert combinations of solvent tolerance genes in microbial genomes and characterised them for an improvement in tolerance.

To sum up, by using synthetic biology tools such as BioBricks, solvent tolerance modules were tested individually and in combination. Fluorescence and luminescence-based systems were constructed by putting together other BioBrick parts which provided novel insights into the possible mechanisms of toxicity of organic solvents. Further work is required to unravel the complexity of the *n*-butanol tolerance phenotype and such work should focus on understanding the exact mechanisms by which individual genes increase tolerance to organic solvents. When individual tolerance modules are properly characterised, it will then be possible to assemble a functional network of solvent tolerance genes. When this is done, solvent production pathways should be coupled to tolerance systems in order to increase the yield and titre of the final products.

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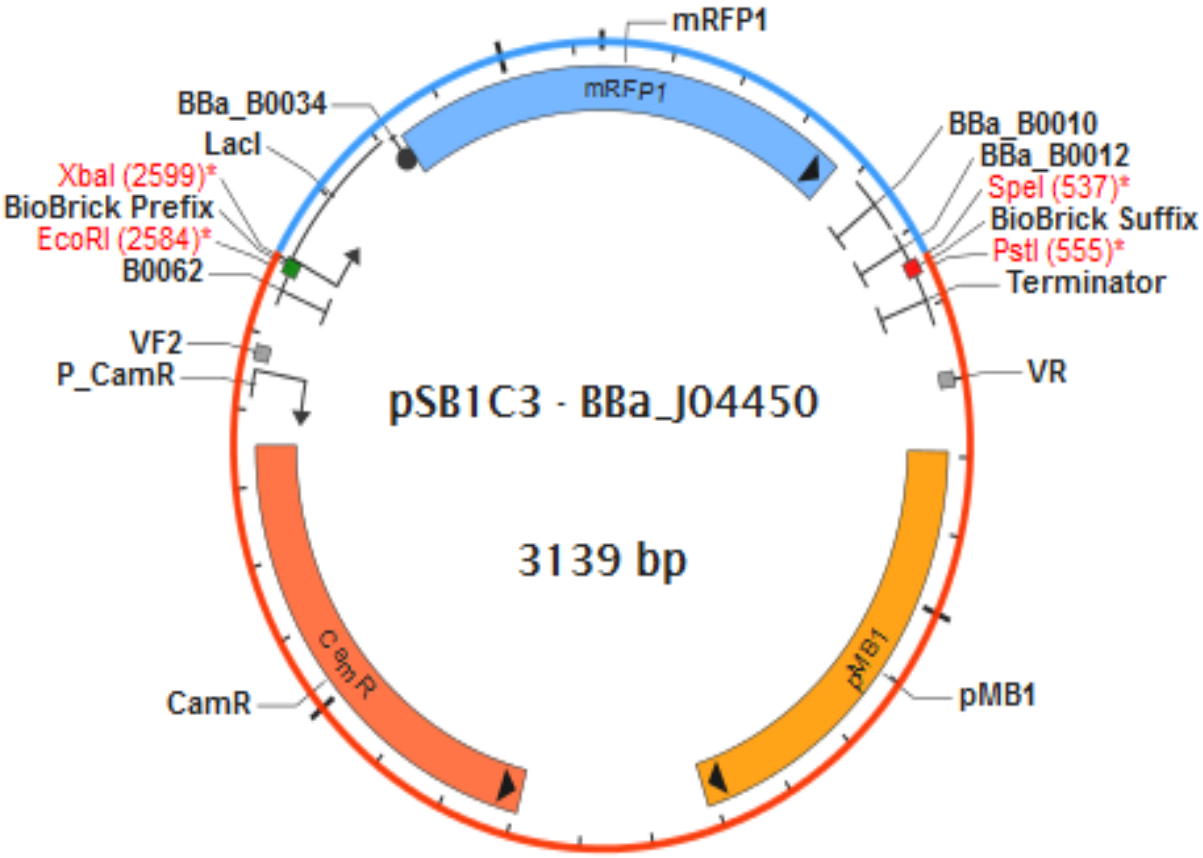
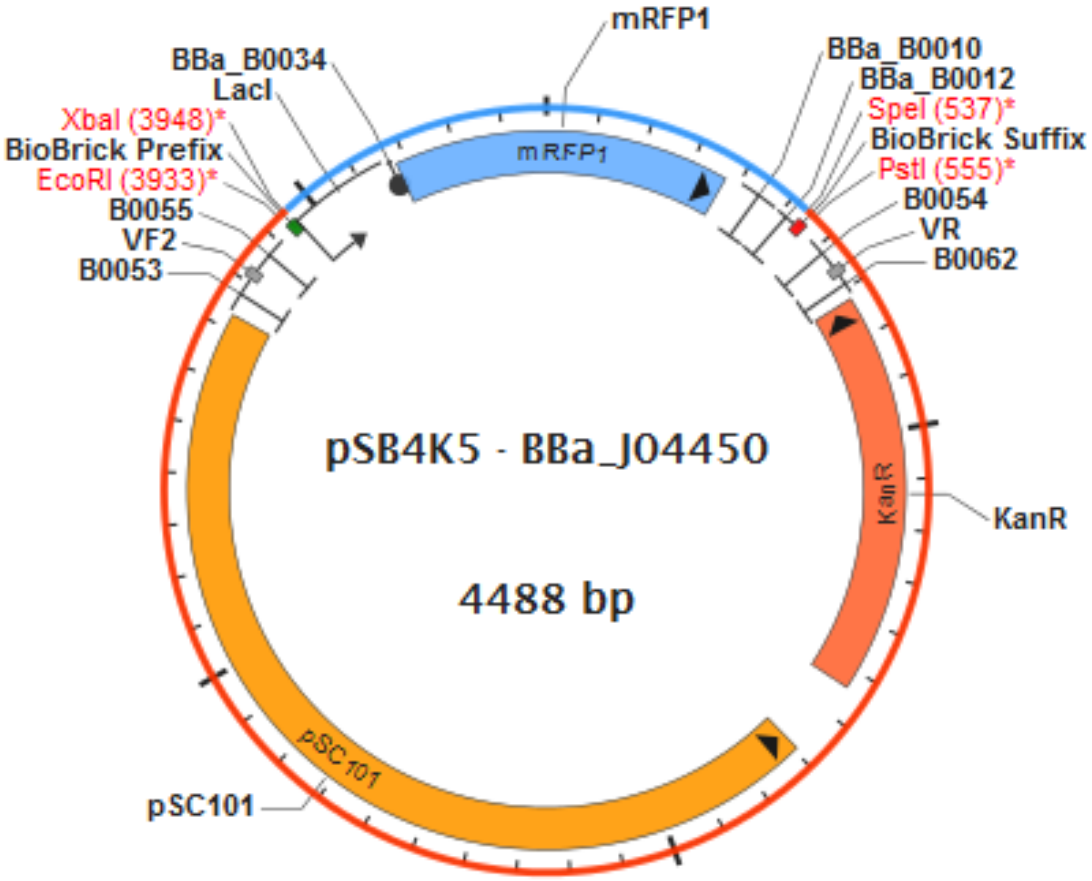
Appendices

Appendix I – Plasmid map of pSB1C3



Source: www.parts.registry.org

Appendix II – Plasmid map of pSB4K5



Source: www.parts.registry.org

Appendix III – Codon optimised sequence of the fatty acid *cis-trans* isomerase (*cti*) gene

EcoRI *XbaI* RBS(BBa_B0034) start

*GAATTC*CTTCTAGAGAAAGAGGAGAAATACTAGATGGTGCATCGTATTCTGGCAGGC
GCATTTGCACTGCTGATTAGCGGTGCAGTTTTTGGTCAGGCACCGCAGAGCAGTCCG
GCAATTAGCTATACCCGTGATATTCAGCCGATCTTTACCGAAAAATGTGTTGCATGT
CATGCCTGTAATGATGCAGCATGTCAGCTGAAACTGGAAAGTCCGGAAGGTGCAGTT
CGTGGTGCAAGCAAAGTTCCGGTTTATCAGGGTGAACGTAGCAAAGCAGTTCCGACC
ACCCGTCTGTTTTATGATGCACATAGCGAAGAACAGTGGCGCAAAAAAGGTTTTTAT
AGCGTTCTGGATAATCAGGGTGGCCAGGCAGCACTGATGGCACGTATGCTGGAACTG
GGCCATAAAACACCGCTGACCCCGAATGCAAAACTGCCGGAAGAAATTGTTCTGGGT
CTGAGCCGCAATAATATGTGTCCGCTGCCGCATGAATTTGATGCATATGCCGGTGCA
CATCCGAAAGAAGGTATGCCGCTGGCAGTTACCGGTCTGACCGATGATGAATATGCA
ACCCTGCGTCGTTGGCTGGCAGCGGGTGCACCGGTTGAATATCAGCCGATTCAGCCG
TCAGCAGCAGAAGCACGTCAGATTGCAGAATGGGAAGAACTGCTGAATCGTCCGGGT
AGCACCGAAGCACTGGTTGGTCGCTGGCTGTATGAACACCTGTTTCTGGCACATATC
TATTTTGCCGGTGGTGAACAGGGTCATTTTTTTCAGTGGGTTCGTAGCCGTACCCCG
AGCGGTAAACCGGTTGATATTATCGCAACCCGTCGTCCGAATGATCCGCCTGGCACC
GATTTCTATTATCGTCTGATTCCGGTTCAGGGTGTGATTGTTCATAAAACCCATATT
ACCTATCCGATGGGTCCGCAGAAACTGAAACGTGTTAAACAACTGTTCTATGCGGGT
GATTGGCATGCAGCAGCACTGCCTGGTTATGGTCCGCGTCATCGTGCAAATCCGTTT
GAAACCTTTGAAGCAATTCCTGCCGTTGCCCGTTATCAGTTTATGCTGGATAATGCC
GAATATTTTGTGCGCACCTTTATTCGTGGTCCGGTTTGTCGTGGCCAGATTGCAACC
GATGTTATTCGTGATAACTTTTGGGCACTGTTTCAAGAACCGGCATTTGATCGTTAT
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ATTGATGATGTTGGTAGCGTGCTGAGCCTGTGGCATGCATATCGTGATAAACGTAAC
GATTATGAAAAACTGCGTCGTGAAGCATATGCAGAAATGCCTGCACCGCGTTGGAGC
ACCCTGTGGGCAGGTAATGATAATGCCCTGCTGAGCATTTTTCGTCATTTTGATAGC
GCAAGCGTTACCAAAGGTCTGGTTGGTGATGTTCCGCTGACCGTTTGGCTGTTTGAT
TATCCGCTGTTTGAACGTACCTATTATCAGCTGGCCGTTAACTTTGATGTGTATGGT
AATGTTAGCCATCAGCTGCAAACACGCCTGTATTTTGATCTGATTCGTAATGGTGCC
GAAGTGAATTTTCTGCGTCTGATGCCTGCCGATCAGCGTAAAGCAATTCTGGGCGAT
TGGTATCAGAATAGCGGTAAAGTTAAAATGTGGATGGATTACGAGGATATCGATACC
GATACCCCGTCAGGTATTAAACTGGATCCGCGTAATCCGAAACGTGATTTTGGTCTG
AAACTGCTGCAACGTACCGGTAGCCTGAATGCAGCACCGGATCCGATTAACCGTTGT
CAGGGTGCATTTTGTAGCCGTCCGCAGATGAGCGAAGAATTTCGTAATGCAGAACAG
AGCCTGAGCCGTCTGGTGAGCCGTCCGGCAGCAGGTCTGAAAGTTATTAATCAGCTG
CCTGAAGCAACCATGCTGCGTATTGAAGGTCAGGATGGTCAGCGTCAGGTTTATAGC
CTGCTGCGTAATCGTGCCCATAGCAATGTTGCCTTTCTGCTGGGTGAAGCCTATCGT
TATCAGCCTGGTCTGGATACCCTGACCCTGTATCCGGGTGTTCTGAGCAGCTATCCG
AATTTTATCTTTAACATCCCGACCAAAGATGTGCCGGAATTTGTTGAAGATATGGAA
TATGCCAAAGACGACGCAGCCAAATTTGAACGCATTGTTATGCGTTGGGGTGTTCGT
CGTAGCCATCCGGCATTTTGGCGTTATTTTCATGATCTGAACAGCTATATCAAAGAA
ACCGAACCGGTGGAAGCCGGTGTGCTGGATATGAATCGTTATGAAAATCTGTAATAC
TAGTACCTGCAG

Stop

SpeI *PstI*